Structure/Function Analysis of the Interaction of Adenomatous Polyposis Coli with DNA Polymerase β and Its Implications for Base Excision Repair[†]

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ABSTRACT: Mutations in the adenomatous polyposis coli (*APC*) gene are associated with an early onset of colorectal carcinogenesis. Previously, we described a novel role for the APC polypeptide in base excision repair (BER). The single-nucleotide (SN) and long-patch (LP) BER pathways act to repair the abasic sites in DNA that are induced by stressors, such as spontaneous oxidation/reduction, alkylation, and hyperthermia. We have shown that APC interacts with DNA polymerase β (Pol-β) and flap endonuclease 1 (Fen-1) and blocks Pol-β-directed strand-displacement synthesis. In this study, we have mapped the APC interaction site in Pol-β and have found that Thr79, Lys81, and Arg83 of Pol-β were critical for its interaction with APC. The Pol-β protein (T79A/K81A/R83A) blocked strand-displacement DNA synthesis in which tetrahydrofuran was used as DNA substrate. We further showed that the APC-mediated blockage of LP-BER was due to inhibition of Fen-1 activity. Analysis of the APC-mediated blockage of SN-BER indicated that the interaction of APC with Pol-β blocked SN-BER activity by inhibiting Pol-β-directed deoxyribose phosphate lyase activity. Collectively, our findings indicate that APC blocked both Pol-β-directed SN- and LP-BER pathways and increased sensitivity of cells to alkylation induced DNA damage.

Mammalian cells use DNA repair mechanisms to maintain the integrity of their genome (*1*). Among these mechanisms, the base excision repair (BER)[1] pathway protects the genome by removing the damaged nucleotides, abasic sites, and single-strand DNA breaks that occur upon exposure to a variety of exogenous and endogenous stresses (*2*). In mammalian cells, BER can proceed through at least two pathways that are distinguished on the basis of the repair patch size as well as by the contributions of different proteins involved in the pathway (*3*, *4*). These two pathways are described as the single nucleotide (SN) BER and long patch (LP) BER pathways. In both pathways, repair is initiated by removal of the modified base, which results in the generation of an abasic site (AP-site). The removal of the modified base can occur either spontaneously or by a damage-specific DNA glycosylase. There are two types of DNA glycosylases: monofunctional and bifunctional. Monofunctional DNA glycosylases cleave only the glycosidic bond and then protect the abasic site until apurinic/apyrimidinic (AP) endonuclease 1 (APE-1) cleaves the DNA backbone 5′ to the AP-site, generating a 3′-OH and a 5′-deoxyribose phosphate (dRP) residue (*5*). The bifunctional DNA glycosylases have additional AP-lyase activity that incises the AP-site on the 3′-side, generating a 3′-sugar−phosphate residue and a 5′-phosphate (*5*). The 5′-dRP residue is removed by the dRP-lyase activity of DNA polymerase β (Pol-β) to yield a 5′-phosphorylated single-nucleotide gap (*6*). Pol-β then fills the gap, and DNA ligase I or III seals the nick (*4*). This repair process is shunted into an alternative route, however, when the AP-site or dRP residue becomes oxidized or reduced. In this case, the modified dRP group does not serve as a substrate for the lyase activity of Pol-β; rather, Pol-β-dependent strand-displacement DNA synthesis generates a longer repair patch and downstream 5′-overhang of a single-stranded DNA with a modified sugar at its 5′-end. The 5′-overhang DNA flap is cleaved by flap endonuclease 1 (Fen-1), and finally the nick is sealed by DNA ligase I or III (*7*−*10*).

Germ-line mutations of the adenomatous polyposis coli (*APC*) tumor suppressor gene invariably result in familial adenomatous polyposis coli (FAP), a syndrome characterized by early onset of the development of colorectal cancer (*11*). The *APC* gene product is composed of 2843 amino acids and has a molecular mass of approximately 310 kDa. Most

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[1] Abbreviations: APC, adenomatous polyposis coli; APE, apurinic/apyrimidinic endonuclease; BER, base excision repair; DRI domain, DNA repair inhibitory domain; dRP-lyase, deoxyribose phosphate lyase: Fen-1, flap endonuclease 1; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; LP-BER, long-patch base excision repair; PCNA, proliferating cell nuclear antigen; SN-BER, single-nucleotide base excision repair; Pol-β, DNA polymerase β.

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somatic mutations are clustered between codons 1284 and 1580, also called the mutator cluster region (MCR) (*12*−*15*). APC is a member of the Wnt signaling pathway, and one of its known functions is to regulate the level of β-catenin. Alterations in β-catenin regulation are very common in human tumors (*16*, *17*). Loss of APC activity is associated with the stabilization of cytosolic β-catenin that ultimately migrates to the nucleus and activates a cascade of events leading to tumorigenesis. APC also interacts with a multitude of other cellular proteins, including axin-2 (*AXIN2*), plakoglobin (*JUP*), Asef (*ARHGEF4*), kinesin superfamily-associated protein 3 (*KIFAP3*), EB1 (*MAPRE1*), microtubule proteins, and the human homologue of *Drosophila* discs large (*DLG1*). These interactions suggest that APC potentially can regulate many cellular functions, including aberrant growth in many ectodermally derived squamous epithelia, embryonic cell fate, intercellular adhesion, cytoskeletal organization, regulation of plakoglobin levels, regulation of the cell cycle and apoptosis, orientation of asymmetric stem cell division, chromosomal segregation, and cell polarization (*18*−*22*). In addition to these known biological functions of APC, our recent findings indicate that APC can regulate BER (*23*−*25*).

Pol-β, which is the smallest eukaryotic DNA polymerase, plays a central role in BER. It is a 39-kDa protein that consists of an 8-kDa amino-terminal domain with dRP-lyase and 5′-phosphate recognition activities and a 31-kDa carboxyl-terminal domain with nucleotidyltransferase activity (*26*). The crystal and solution structures of the amino-terminal 8-kDa lyase domain (amino acids 1−87) have been determined (*27*, *28*). This domain is composed of two pairs of antiparallel α-helices and possesses the dRP-lyase activity. The lyase domain also contains a motif termed Helix−hairpin−Helix (HhH), which is common in many other DNA repair proteins (*29*). Biochemical (*30*−*32*) and crystallographic (*33*) studies indicate that Lys72 plays a critical role in the lyase reaction mechanism. This reaction proceeds via a Schiff base intermediate between Pol-β and the 5′-dRP residue of the substrate, whereby the side chain of Lys72 provides the nucleophile for the completion of the reaction. However, the involvement of the lyase domain in strand-displacement synthesis of Pol-β has not been clearly defined. Our present communication describes the characterization of the interaction of APC with Pol-β and indicates a role for APC in the regulation of both the SN- and LP-BER pathways. We have mapped the interaction of APC with Pol-β and found that residues Thr79, Lys81, and Arg83 of the linker region of Pol-β protein are critical for the interaction with APC. Interaction of APC with Pol-β blocks strand-displacement DNA synthesis as well as the dRP-lyase activity of Pol-β. Our mutational analysis of Pol-β further confirmed the role of APC on the function of this BER enzyme. These findings describe a novel role of APC in the regulation of both SN- and LP-BER activities and suggest a function for the linker region of Pol-β in BER activity.

EXPERIMENTAL PROCEDURES

Maintenance of Mammalian and Yeast Cell Lines. Human colon cancer cell lines HCT-116-APC(WT) (wild-type APC expression), HCT-116-APC(KD) (knockdown APC expression by pSiRNA-APC), and LS411N (mutant APC expression lacking DRI domain) were grown in McCoy's 5a medium, and LoVo cells (mutant APC expression lacking DRI domain) were grown in Ham's F12 medium at 37 °C under a humidified atmosphere of 5% CO_2. In each case, the medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

The yeast strain PJ69-4A (*MAT*a *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *GAL2*−*ADE2 LYS2*::*GAL1*−*HIS3 met2*::*GAL7*−*lacZ*) was grown on synthetic dropout medium lacking lysine [(0.17% Difco yeast nitrogen base without amino acids, ammonium sulfate (5.0 g/L), complete supplemental amino acid mixture minus appropriate amino acid containing 2% glucose)].

Oligonucleotides and Chemicals. All oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). Restriction enzymes, T4 polynucleotide kinase (PNK), terminal deoxynucleotidyltransferase (TdT) and Vent-DNA polymerase were purchased from New England Biolabs (Ipswich, MA) and the radionuclides [γ-^{32}P]ATP and [α-^{32}P]-ddATP were purchased from MP Biomedicals (Solon, OH) and Amersham Biosciences (Piscataway, NJ), respectively.

Generation of Pol-β Deletion Constructs. Plasmids were constructed to encode various segments of Pol-β (amino acids 60−120, 80−170, 140−200, and 160−250) to identify the amino acids of Pol-β that interact with the DNA repair inhibitory (DRI) domain of APC. These deletion fragments were subcloned into the pGAD-C3 vector between the *Pst*I and *Bam*HI restriction sites. The following primers were used to generate various Pol-β deletion constructs: Polβ(60−120) (sense primer 5′-CGCGGATCCAAGAAATTGCCTGGAGTA-3′ and antisense primer 5′-CCAATGCATTGGTTCTGCAGTTTAATTCCTTCATCTAC-3′), Polβ(80−170) (sense primer 5′-CGCGGATCCGGAAAATTACGTAAACTG-3′ and antisense primer 5′-CCAATGCATTGGTTCTGCAGATCCACTTTTTTAACTT-3′), Polβ(140−200) (sense primer 5′-CGCGGATCCCTGAAATATTTTGGGGAC-3′ and antisense primer 5′-CCAATGCATTGGTTCTGCAGGAAGCTGGGATGGGTCAG-3′), and Polβ(160−250) (sense primer 5′-CGCGGATCCGATATTGTTCTAAATGAA-3′ and antisense primer 5′-CCAATGCATTGGTTCTGCAGATATTCTTTTTTCATCATT-3′).

Site-Directed Mutagenesis of Pol-β. Two different sets of Pol-β mutants, Set-1 mutant (T79A/K81A/R83A) and Set-2 mutant (R89A/Q90A/D92A), were generated by use of the Quick Change site-directed mutagenesis kit from Stratagene (La Jolla, CA). The following primer pairs were used for Set-1 and Set-2 mutants: Set-1, sense primer 5′-GAAAAGATTGATGAGTTTTTAGCAGCCGGAGCGTTAGCTAAACTGGAAAAGATTCGGCAG-3′ and antisense primer 5′-CTGCCGAATCTTTTCCAGTTTAGCTAACGCTCCGGCTGCTAAAAACTCATCAATCTTTTC-3′; Set-2, sense primer 5′-GGAAAATTACGTAAACTGGAAAAGATTGCCGCGGATGCTACGAGTTCATCCATCAATTTCCTG-3′ and antisense primer 5′-CAGGAAATTGATGGATGAACTCGTAGCATCCGCGGCAATCTTTTCCAGTTTACGTAATTTTCC-3′.

Yeast Two-Hybrid Assay. We employed the yeast two-hybrid assay to identify critical amino acids of Pol-β to define the functional interaction with APC in vivo. APC cDNA fragments containing the wild-type (residues 1190−1328) or the mutant DRI domain (residues 1200−1324, in which amino acids Ile1259 and Tyr1262 were replaced with alanine) were fused to the yeast Gal4 DNA-binding domain (BD) in

plasmid pGBDU-C3. The interacting Pol-β protein fragments such as full-length and various segments (residues 60−120, 80−170, 140−200, and 160−250) were fused to the yeast Gal4 activation domain (AD) in plasmid pGAD-C3. Set-1 (T79A/K81A/R83A) and Set-2 (R89A/Q90A/D92A) Pol-β mutants also were cloned into plasmid pGAD-C3. Yeast two-hybrid analysis was performed as described previously (*23*, *25*).

Cloning of Histidine-Tagged Plasmids and Overexpression and Purification of Proteins. Human wild-type *Pol-β* (pWL11-hpolβ) and Set-1 mutant *Pol-β* (T79A/ K81A/R83A) cDNAs were cloned into pET23d vector (carboxyl-terminal hexahistidine tag). The wild-type *Pol-β* (pET23d-Ct-his-hPolβ), mutant *Pol-β* (pET23d-Ct-his-hPolβMut1), *Fen-1* (pET23d-Ct-his-hFen1), and DNA ligase I (pET-his-hDNA Ligase I) plasmids were transferred into BL21(DE3)pLysS cells. The overexpressed proteins were purified to homogeneity according to published protocols with some modifications (*34*−*36*).

In Vitro BER Assays. To study LP- and SN-BER activities, we used two different types of DNA substrates as described earlier (*25*). For LP-BER activity, an AP-site analogue (3-hydroxy-2-hydroxymethyltetrahydrofuran, noted as F) was introduced at the 24th position of the 63-mer DNA (F-DNA). For SN-BER, uracil was introduced at the 24th position of the 63-mer sense oligonucleotide (U-DNA) (*25*). For strand-displacement synthesis, the reaction was reconstituted with purified proteins under the following conditions. The reaction mixture contained 30 mM Hepes, pH 7.5, 30 mM KCl, 8.0 mM $MgCl_2$, 1.0 mM DTT, 100 μg/mL BSA, 0.01% (v/v) Nonidet P-40, 0.5 mM ATP, and 10 μM each dATP, dCTP, dGTP, and dTTP in a final volume of 20 μL. The BER reaction mixture was assembled on ice by the addition of 5 nM Pol-β, 2.5 nM PCNA, and 0.2 nM Fen-1. This mixture was preincubated with APCwt and APC(I-A,Y-A) peptides for 5 min at 22 °C. The amounts of APCwt and APC(I-A,Y-A) peptides used in each experiment are given in the respective figure captions. Strand-displacement synthesis was initiated by the addition of 2.5 nM ^{32}P-labeled F-DNA or U-DNA (precut with 1 nM APE) to corresponding tubes and further incubated for 30 min at 37 °C. For complete BER, 0.4 nM of DNA ligase I was added to the above reaction mixture, which was incubated for 30 min at 37 °C.

In Vitro BER Assay with Nuclear Extract. A high-efficiency nuclear extract (NE) from HCT-116-APC(WT), HCT-116-APC(KD), LS411N, and LoVo cells was prepared by the procedure of Shapiro et al. (*37*). The LP-BER assay was assembled at 37 °C with 5 μg of NE, 2.5 nM ^{32}P-labeled F-DNA (precut with 1 nM APE), and 10 μM each dATP, dCTP, dGTP, and dTTP in a final volume of 20 μL. The reaction was stopped at different time intervals. BER reactions in both assays were terminated by the addition of 20 μL of stop solution [5.0 mM EDTA and 0.4% (w/v) SDS] with 1 μg of proteinase K and 5 μg of carrier tRNA. After incubation for an additional 20 min at 37 °C, the DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) followed by ethanol precipitation. The reaction products were resolved on a 15% polyacrylamide−7 M urea gel.

dRP-Lyase Activity Assay. A 63-mer oligonucleotide containing uracil at the 24th position was labeled at the 3′-end by terminal deoxynucleotidyltransferase with [α-^{32}P]-ddATP and annealed to the complementary oligonucleotide. To remove uracil, the 3′-end-labeled double-stranded oligonucleotide (2.5 nM) was treated with uracil-DNA glycosylase (UDG) (2 units) for 20 min at 37 °C in 20 μL of buffer containing 30 mM Hepes, pH 7.5, 30 mM KCl, 8.0 mM $MgCl_2$, 1.0 mM DTT, 100 μg/mL bovine serum albumin, 0.01% (v/v) Nonidet P-40, and 0.5 mM ATP. After incubation, the mixture was supplemented with 1.0 nM APE and further incubated for 10 min, thus generating the substrate for dRP-lyase activity.

Pol-βWt or Pol-βMut-1 protein (2.5 nM) was preincubated with variable amounts of APCwt or APC(I-A,Y-A) peptides for 5 min at 22 °C. The reaction was initiated by adding these preincubated protein and peptide complexes with dRP-lyase substrate and incubating the reaction mixture at 37 °C for 15 min. After incubation, $NaBH_4$ was added to a final concentration of 340 mM, and the samples were kept on ice for 30 min. The stabilized (reduced) DNA products were ethanol-precipitated in the presence of 5.0 μg of carrier tRNA, and resuspended in 10 μL of a gel-loading buffer [95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromophenol blue, and 0.02% (w/v) xylene cyanol]. After incubation at 75 °C for 2 min, the reaction products were resolved on a 15% polyacrylamide−7 M urea gel.

Plasmid-Based BER in Live Cells. The plasmid-based BER assay in live cells was essentially the same as described in our previous studies (*23*, *24*). Briefly, pGL2-p21, a closed circular DNA of p21(Waf-1/Cip1) promoter containing random C-residues, was deaminated with 3 M sodium bisulfite in the presence of 50 mM hydroquinone. The resulting U-p21P was treated with UDG and then reduced with 0.1 M sodium borohydride to generate reduced AP-sites (R-p21P) for use as an LP-BER substrate. LoVo cells were cotransfected with either pCMV-vector or pCMV-APC plasmids (*25*), along with U-p21P or R-p21P plasmids, by use of Lipofectamine reagent. The HCT-116-APC(WT) and HCT-116-APC(KD) cells were transfected with U-p21P or R-p21P plasmids by use of Lipofectamine reagent. These cells were also cotransfected with the pCMV-β-galactoside (β-gal) plasmid, which served as an internal control to correct for differences in transfection efficiency. After 5 h of transfection (once the cells were acclimated), one set of cells was harvested. The promoter activity in this set of cells was determined at this time point, which was considered to be the zero time point. The medium of the remaining dishes were aspirated and replaced with complete medium supplemented with 10% fetal bovine serum. Cells were harvested at different time intervals, and LP-BER activity was measured by determining the luciferase gene-reporter activity of cellular lysates by use of a Moonlight 3010 Illuminometer (Promega, San Diego, CA).

MMS Toxicity Assay. To determine the MMS toxicity, a clonogenic assay was performed with HCT-116-APC(WT) and HCT-116-APC(KD) cell lines. Cells were trypsinized and a single cell suspension was prepared. Cells were plated at a density of 100 cells/35 mm well. Cells were treated with different concentrations of methylmethane sulfonate (MMS). After 72 h, the medium was replaced with a fresh medium. Cells were allowed to grow for a further 8 days and then stained with 0.025% crystal violet. The excess crystal violet was removed with 30% methanol, plates were air-dried at room temperature, and numbers of colonies were counted.

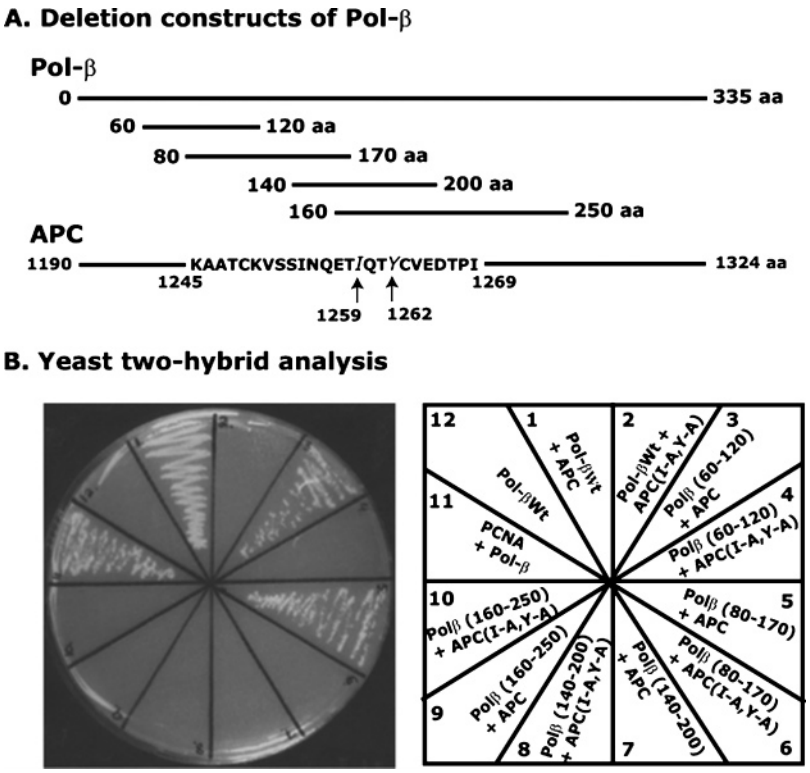

FIGURE 1: Yeast two-hybrid analysis of the interaction of APC with Pol-β. The yeast two-hybrid constructs are described under Experimental Procedures. (A) Deletion constructs of Pol-β: APCwt and APC(I-A,Y-A) plasmids used in the yeast two-hybrid analysis. The positions of mutated isoleucine (I) and tyrosine (Y) amino acids are shown in italic type and indicated with arrows in the diagram. (B) Results of the yeast two-hybrid analysis of the interaction of APC with deletion constructs of Pol-β. Yeast PJ69-4A cells were cotransformed with pGBDU-C3-APCwt (amino acids 1190−1328) or pGBDU-C3-APC(I-A,Y-A) (amino acids 1200−1324; I1259A, Y1262A) plasmids with either pGAD-C3-Pol-βWt or different deletion construct plasmids. For a positive control, the PCNA/Pol-β interaction is shown. Data are representative of three different experiments.

RESULTS

APC Binds with the Linker Region 8-kDa Domain of Pol-β. In previous studies, we showed that APC interacts with Pol-β (*23*, *25*). While the interaction domain of APC with Pol-β was fully characterized (*25*), the domain of Pol-β that interacts with APC was not identified. In order to map the critical region of Pol-β necessary for its interaction with APC, deletion constructs of Pol-β were made through polymerase chain reaction (PCR) amplification and cloned into the pGAD-C3 vector. These constructs were then used in a yeast two-hybrid analysis with a plasmid expressing wild-type APC, pGBDU-C3-APCwt, or a plasmid expressing a mutant APC, pGBDU-C3-APC(I-A,Y-A), that did not bind Pol-β (Figure 1A). We found an interaction of APCwt but not of APC(I-A,Y-A) with Pol-βWt (Figure 1B, compare slices 1 and 2). The interaction of Pol-βWt with PCNA served as a positive control (Figure 1B, slice 11), and the results obtained were consistent with previous findings (*38*). Pol-βWt alone was used in the assay to determine the background growth of the yeast cells (Figure 1B, slice 12). Positive interaction of wild-type APC was observed with the Polβ (60−120) and Polβ (80−170) constructs (Figure 1B, slices 3 and 5, respectively). Other Pol-β constructs, such as Polβ (140−200) and Polβ (160−250), did not show an interaction with APCwt. These results suggested that the interaction domain of Pol-β with APC was located within the stretch of amino acid residues 80−120.

To further identify critical residues of Pol-β that might be involved in the interaction with APC, we examined the solvent surface accessibility of the residues implicated in the interaction with APC by the yeast two-hybrid analysis to interact with APC. Since the crystal structure of APC has not been solved, it was not feasible to identify probable interactions through possible docking modes. The crystal structure of a substrate complex of Pol-β indicates that it is composed of two domains with distinct enzymatic activities necessary for SN-BER: an amino-terminal lyase domain and a carboxyl-terminal polymerase domain (Figure 2A) (*26*). This figure was made by use of UCSF Chimera (*39*). The residues suspected of interacting with APC are in a stretch of amino acids (80−120) that connects these domains. From the structure of the ternary substrate complex (*40*, *41*), two regions—Set-1 (amino acids Thr79, Lys81, and Arg83) and Set-2 (amino acids Arg89, Gln90, and Asp92)—were identified that exhibited high solvent accessibility (Figure 2B,C). The protein backbone of this region was observed in several conformations depending on the liganded states (with metals, dNTP, or DNA) of Pol-β (*26*). Alteration of the backbone dynamics of this region can be expected to affect Pol-β-dependent substrate binding and/or catalysis. Next, we changed the Set-1 and Set-2 amino acids to alanine (A) by site-directed mutagenesis and examined their role in the interaction with APC in a yeast two-hybrid analysis. Appropriate positive and negative controls such as the interaction of PCNA and Pol-β (Figure 3, slice 4) and the APCwt plasmid alone (Figure 3, slice 5), respectively, were run to validate our assay conditions. The results showed that the Set-1 mutant (Pol-βMut-1) abolished the interaction of Pol-β

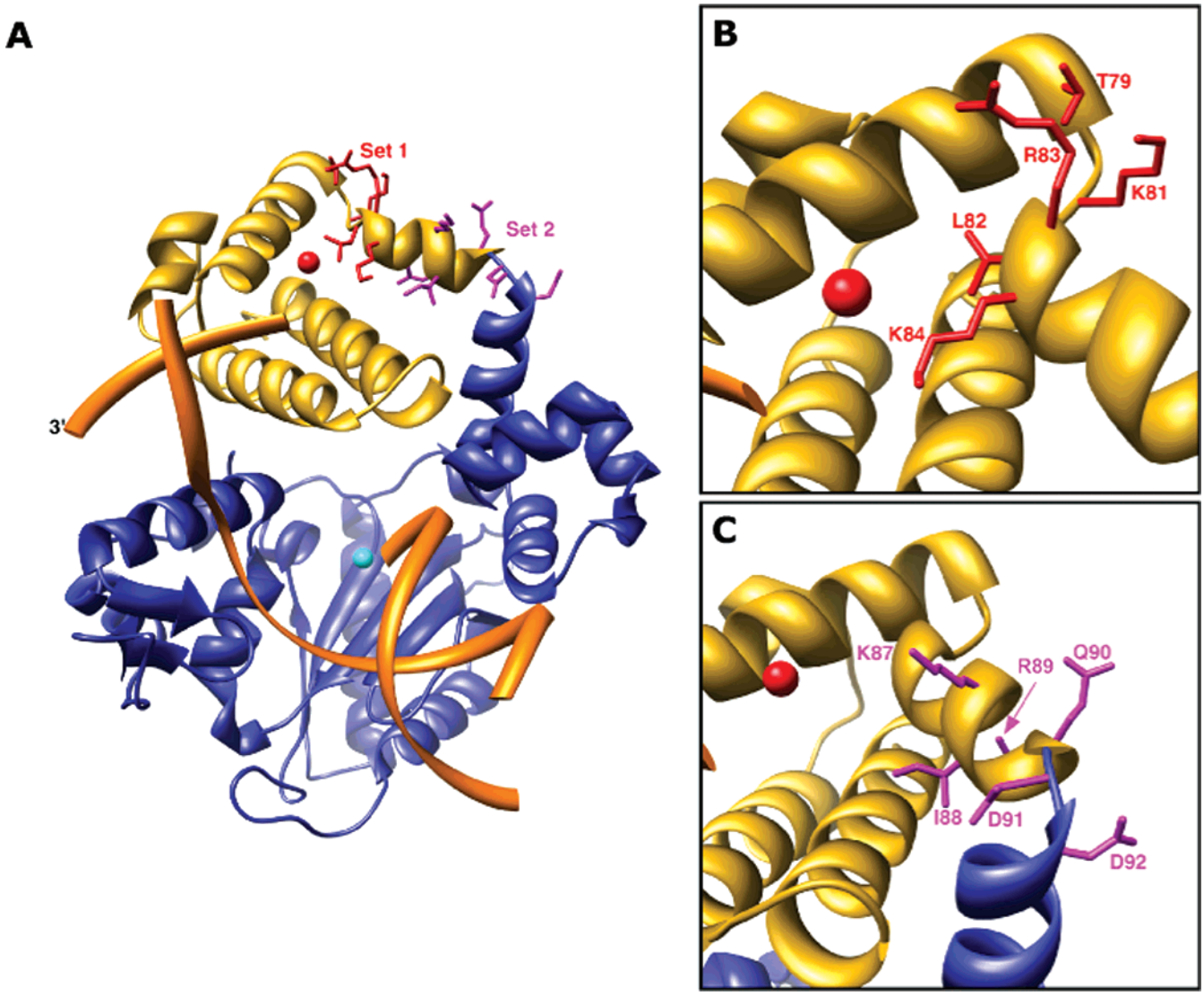

FIGURE 2: Ribbon representation of Pol-β highlighting the position of key mutant sets. (A) Set-1 (red) and Set-2 (magenta) residues are displayed on a ribbon representation of a ternary substrate complex of Pol-β (PDB accession code 2FMS). The lyase and polymerase domains are colored gold and blue, respectively, and the DNA backbone is orange. Additionally, a light blue sphere (catalytic Mg^{2+}) identifies the polymerase active site and a red sphere (NZ of Lys72) identifies the dRP-lyase active site. The 3′-end of the downstream gapped DNA strand also is indicated. (B) Set-1 (residues 79−84) side chains; (C) Set-2 (residues 87−92) side chains.

with APC (Figure 3, slice 3); however, the mutations in Set-2 (Pol-βMut-2) showed no effect (Figure 3, slice 2). From these results it became clear that the amino acid residues Thr79, Lys81, and Arg83 of Pol-β were critical for the interaction with APC and thus could play an important role in the mechanism by which APC blocks Pol-β activity.

Pol-βMut-1 Mimics APC-Dependent Inhibition of Strand-Displacement Synthesis of LP-BER. In previous studies, we showed that APC blocks strand-displacement synthesis and that amino acid residues Ile1259 and Tyr1262 of APC were important for this activity (*23*, *25*). On the basis of our data indicating that amino acid residues Thr79, Lys81, and Arg83 of Pol-β determined the site of interaction of Pol-β with APC, we hypothesized that these amino acid residues play a role in the APC-mediated blockage of Pol-β-directed strand-displacement synthesis.

To test this hypothesis, we determined whether mutated Pol-β can mimic the effect of APC on strand-displacement synthesis of LP-BER in a reconstituted in vitro BER assay system with ^{32}P-F-DNA as the substrate. In these experiments, we used purified His-tagged Pol-βWt and Pol-βMut-1 (T79A/K81A/R83A) proteins (Supporting Information Figure S1). Strand-displacement synthesis was seen in the system reconstituted with the Pol-βWt protein, and the synthesis occurred in a time-dependent (Supporting Information Figure S1B, compare lane 2 with lanes 3−11) and concentration-dependent manner (Supporting Information Figure S2B, compare lane 2 with lanes 3−5), confirming our previously published data. Mutation of the Set-1 residues completely abolished strand-displacement synthesis, with the inhibition being both time-dependent (Supporting Information Figure S1B, compare lanes 3−11 with lanes 12−20) and concentration-dependent (Supporting Information Figure S2B, compare lanes 3−5 with lanes 6−8). The single-nucleotide incorporation activity of the Pol-β with ^{32}P-F-DNA was unaffected by these mutations; however, it was similar to the effect of APC on single-nucleotide incorporation activity as shown in our earlier studies (*23*, *25*). Taken together, these results suggested that the Set-1 mutant abolished the physical interaction of Pol-β with APC and blocked its strand-

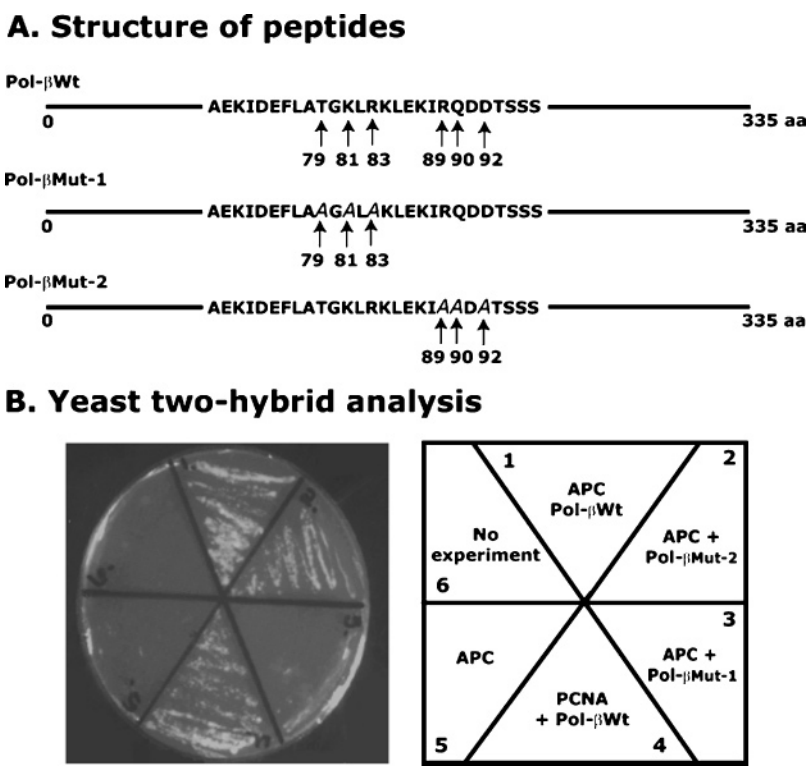

FIGURE 3: Yeast two-hybrid analysis of critical residues of Pol-β involved in the interaction with APC. (A) Yeast two-hybrid constructs prepared by site-directed mutagenesis at the Set-1 and Set-2 amino acids. These are shown in italic type and indicated with arrows in the diagram. (B) Interaction of APC with Set-1 and Set-2 Pol-β mutant plasmids. Yeast PJ69-4A cells were cotransformed with pGBDU-C3-APCwt plasmid (residues 1190−1328) and with pGAD-C3-Pol-βWt, pGAD-C3-Pol-βMut(Set-1), or pGAD-C3-Pol-βMut(Set-2) plasmids. For a positive control, PCNA/Pol-β interaction is shown. The data presented are representative of three different experiments.

displacement synthesis. Since the strand-displacement activity of Pol-β is not involved in SN-BER, and LP-BER requires Fen-1 regardless of this activity, the significance of the intrinsic strand-displacement activity is not clear.

Pol-βMut-1 Does Not Block the Repair of F-DNA. Next, we tested whether the blockage of strand-displacement synthesis by Pol-βMut-1 was sufficient to block LP-BER. For these experiments, we assembled the complete LP-BER assay as outlined in Figure 4A. The results showed a Fen-1-dependent increase in the Pol-β-directed strand-displacement synthesis (Figure 4B, compare lanes 3 and 4). Complete DNA repair was observed in the presence of DNA ligase I (Figure 4B, lane 5; see formation of the 63-mer ligated product), which was blocked in the presence of APCwt peptide but not with the APC(I-A,Y-A) peptide in a dose-dependent manner [Figure 4B, compare lane 5 with lanes 6−8 for APCwt and with lanes 9−11 for APC(I-A,Y-A), respectively, for formation of the 63-mer ligated product]. When determined with the Pol-βMut-1 protein, Fen-1 partially relieved the blockage of Pol-βMut-1-directed strand-displacement synthesis and stimulated two-nucleotide incorporation (Figure 4B, compare lanes 12 and 13) as compared to the six-nucleotide incorporation with Pol-βWt (Figure 4B, compare lanes 3 and 4). The two-nucleotide strand-displacement synthesis by Pol-βMut-1 protein was sufficient to carry out LP-BER in the presence of Fen-1. Furthermore, complete DNA repair was observed with Pol-βMut-1 in the presence of DNA ligase I (Figure 4B, lane 14; see formation of the 63-mer ligated product). However, repair activity with Pol-βMut-1 was less efficient than with Pol-βWt (Figure 4B, compare lanes 14 and 5). These results indicate that Pol-βMut-1 can process F-DNA, but it does so in a manner that differs from Pol-βWt. Since APC did not bind with Pol-βMut-1, we expected that the APCwt peptide would not affect the blockage of LP-BER. However, we found that APCwt, but not the APC(I-A,Y-A) mutant peptide, blocked LP-BER (Figure 4B, compare lane 14 with lanes 15−17 and 18−20, respectively). In earlier studies, we showed that APC interacted with Fen-1 and blocked its 5′-flap endonuclease and 3′−5′ exonuclease activities in addition to strand-displacement synthesis (*23*). Since Fen-1 activity represents an important step in the completion of LP-BER with F-DNA, these data suggested that the APC-mediated blockage of LP-BER in the presence of Pol-βMut-1 was due to blockage of Fen-1 activity.

APC-Dependent Blockage of BER Activity with F-DNA Is Mediated through Fen-1. From the above experiments, it has been clear that Pol-βMut-1 did not completely block strand-displacement synthesis in the presence of Fen-1 and that it could support LP-BER. Thus, to further determine the role of Fen-1 in the APC-mediated block of LP-BER by Pol-βMut-1, we assembled the LP-BER assay. As expected, there was complete repair of ^{32}P-F-DNA when Pol-βWt, Fen-1, and DNA ligase I were added together (Supporting Information Figure S3B, lane 5). Complete repair was not accomplished in the absence of Fen-1, however (Supporting Information Figure S3B, lane 6). When we determined the LP-BER activity of the Pol-βMut-1 protein with ^{32}P-F-DNA, a similar result was obtained: complete repair of ^{32}P-F-DNA was observed when Fen-1 was added together with Pol-βMut-1 and DNA ligase I (Supporting Information Figure S3B, lane 9) but was not observed in the absence of Fen-1

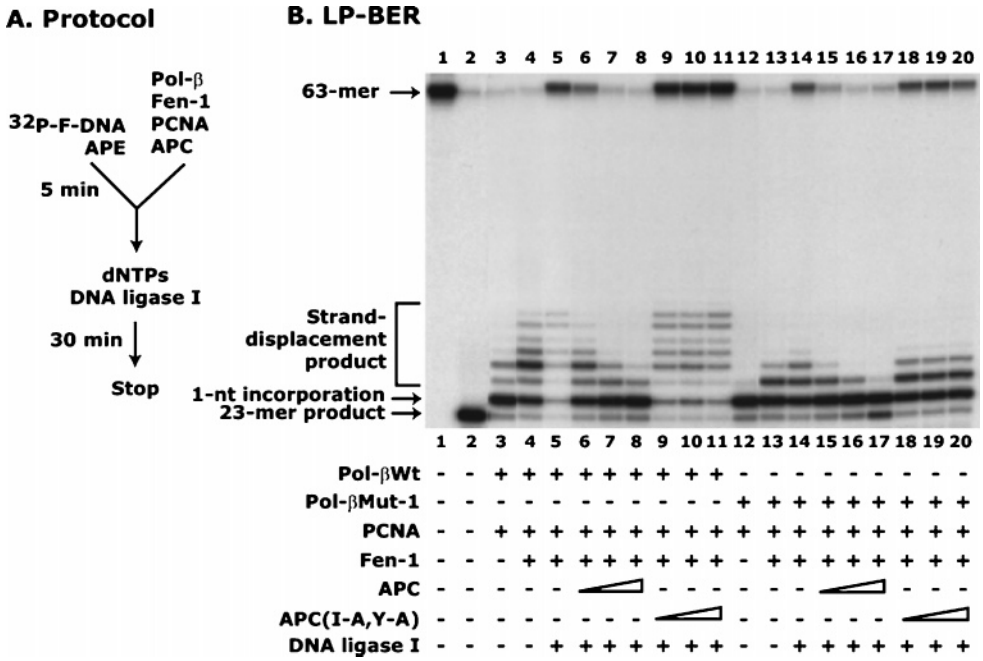

FIGURE 4: Repair of F-DNA in the presence of Pol-βWt and Pol-βMut-1. (A) Schematic representation of the protocol. (B) Effect of Pol-βWt and Pol-βMut-1 proteins on BER activity in the presence and absence of APCwt and APC(I-A,Y-A) peptides. Lanes 6−8 and 15−17 contained 0.25, 0.50 and 1.0 μM APCwt peptides, respectively. Lanes 9−11 and 18−20 contained 0.25, 0.50, and 1.0 μM APC-(I-A,Y-A) mutant peptide, respectively. Lane 1, ^{32}P-labeled 63-mer F-DNA; lane 2, 23-mer product after APE incision. The data presented are representative of three different experiments.

(Supporting Information Figure S3B, lane 10), indicating the role of Fen-1 in APC-mediated blockage of the repair of ^{32}P-F-DNA.

APC Blockage of BER Activity with U-DNA Is Independent of Fen-1 Activity. We next examined whether APC will affect SN-BER and whether the Set-1 residues of Pol-β will be involved in this pathway. Our previous work indicated that APC peptide partially blocked SN-BER (*25*); however, these assays contained Fen-1, which was inhibited by APC but not essential for SN-BER. Therefore, we revisited the question of whether APC inhibited SN-BER directly by performing experiments in the absence of Fen-1. The ^{32}P-U-DNA substrate used in these experiments could be processed by the SN-BER pathway in the absence of Fen-1 and by the LP-BER pathway in the presence of Fen-1 (*43*−*45*). In this study, we were interested specifically in whether APC blocks SN-BER. We reconstituted the SN-BER assay system in the absence of Fen-1 with purified proteins and ^{32}P-U-DNA. Prior to the reaction, the uracil was removed with uracil-DNA glycosylase (UDG) to generate an AP-site, APE was used to 5′-incise the AP site, and the resulting 5′-phosphate/sugar was released as a 5′-dRP moiety by the dRP-lyase activity of Pol-β (Figure 5A). Under these assay conditions with Pol-βWt, we found single-nucleotide incorporation, which was ligated to the 63-mer repair product in the presence of DNA ligase I (Figure 5B, compare lane 2 with lanes 3 and 4). Thus, ^{32}P-U-DNA can be repaired through the SN-BER pathway in the absence of Fen-1. The SN-BER activity was blocked in a dose-dependent manner by addition of the APCwt peptide (Figure 5B, compare lane 4 with lanes 5−7), but not by the addition of the APC(I-A,Y-A) peptide (Figure 5B, compare lane 4 with lanes 8−10), suggesting that APC can block the SN-BER-mediated repair of ^{32}P−U-DNA. The repair of ^{32}P−U-DNA in the presence of Fen-1 that was mediated by the LP-BER pathway also was blocked by the addition of APCwt (data not shown).

Next, we determined the mechanism by which APC might be involved in the blockage of the repair of ^{32}P-U-DNA by the SN-BER pathway. Since APC interacted with the Set-1 amino acids of Pol-β (Thr79, Lys81, and Arg83), we used the Set-1 mutant Pol-β protein (Pol-βMut-1) to mimic the effect of APC. The single-nucleotide incorporation activity of the Pol-βMut-1 protein using ^{32}P-U-DNA as the substrate was similar to that of the Pol-βWt protein (Figure 5B, compare lane 3 and 11, respectively). Analysis of the effect of Pol-βMut-1 on the complete repair reaction in the presence of DNA ligase I indicated that Pol-βMut-1 did not block the repair of ^{32}P-U-DNA by the SN-BER pathway (Figure 5B, lane 12). Next, we determined whether APC had any effect on the Pol-βMut-1-directed SN-BER. As APC did not interact with the Pol-βMut-1 protein, we did not expect to observe a block of the Pol-βMut-1-directed SN-BER. The results supported this hypothesis. Inhibition of the Pol-βMut-1-directed SN-BER after addition of either APCwt or APC-(I-A,Y-A) peptides (Figure 5B, compare lanes 13−15 with lanes 16−18) was not observed. Taken together, these results suggested that Pol-βMut-1 retained dRP-lyase activity and supported SN-BER in the absence of Fen-1.

APC Blocks SN-BER by Blocking dRP-Lyase Activity. The blockage of LP-BER by APC can be explained fully by the blockage of Fen-1 activity; however, this mechanism fails to account for the APC-mediated blockage of the SN-BER activity. As it has been well-established that dRP-lyase activity is a rate-limiting step in SN-BER, we investigated whether APC affected dRP-lyase activity. For these experiments, we used a 3′-end-labeled 63-mer U-DNA substrate as described under Experimental Procedures. Once the U-DNA was treated with UDG and APE, it generated a dRP-lyase substrate (40-mer with 5′-dRP). This 5′-dRP moiety is then cleaved by the dRP-lyase activity of Pol-β to form

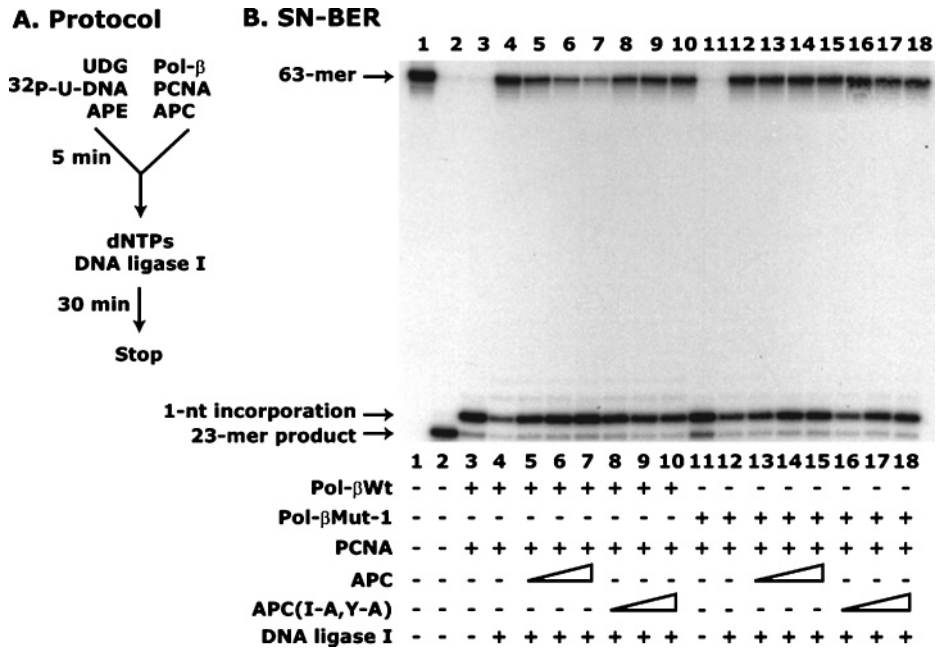

FIGURE 5: Analysis of the role of APC on Pol-βWt and Pol-βMut-1-directed BER with U-DNA. (A) Schematic representation of the protocol. (B) Effect of Pol-βWt and Pol-βMut-1 proteins on the BER activity. Lane 1 shows ^{32}P-labeled 63-mer U-DNA and Lane 2 represents the 23-mer product after APE incision. The data presented are representative of three different experiments.

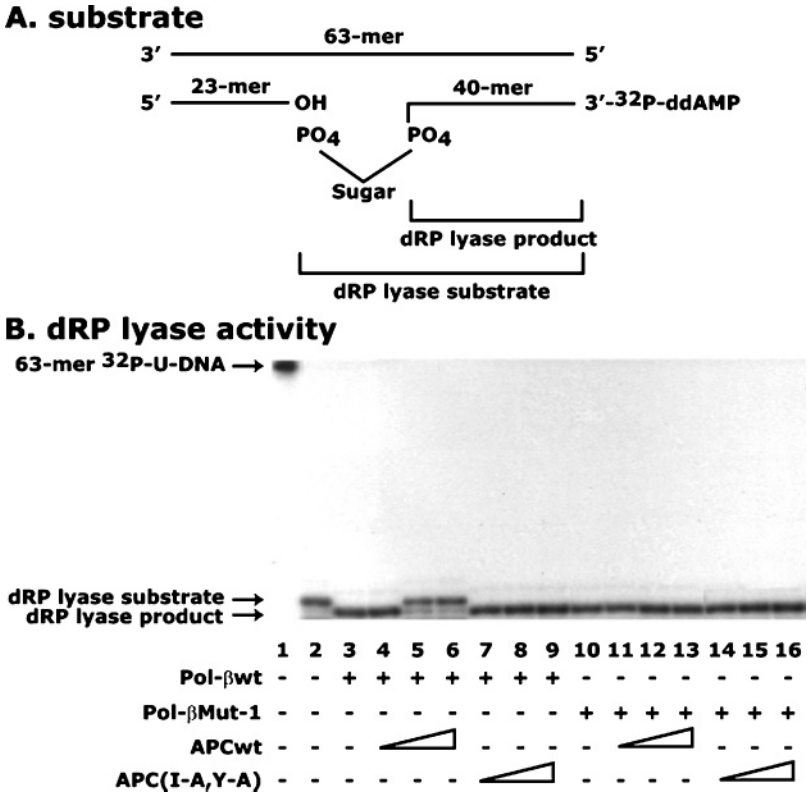

FIGURE 6: Analysis of the effect of APC on Pol-β-directed dRP-lyase activity. (A) Schematic representation of dRP-lyase DNA substrate and its activity. (B) Autoradiogram illustrating the dRP-lyase activity of Pol-βWt and Pol-βMut-1 proteins. The data presented are representative of three different experiments.

the dRP-lyase product (40-mer with 5′-phosphate) (Figure 6A). First, we determined the effect of APCwt on the dRP-lyase activity of Pol-βWt. The results showed efficient dRP-lyase activity (Figure 6B, compare lanes 2 and 3). This activity was blocked by the APCwt peptide in a dose-dependent manner (Figure 6B, compare lane 3 with lanes 4−6) but was unaffected by APC(I-A,Y-A) (Figure 6B, compare lane 3 with lanes 7−9). We then determined the effect of Pol-βMut-1 on its dRP-lyase activity. The results showed that Pol-βMut-1 has dRP-lyase activity (Figure 6B, lane 10). APCwt and APC(I-A,Y-A) peptides did not show any effect on the dRP-lyase activity of Pol-βMut-1 (Figure 6B, compare lane 10 with 11−13 and 14−16, respectively). From these results, we concluded that APC blocks SN-BER by blocking the dRP-lyase activity of the Pol-β protein.

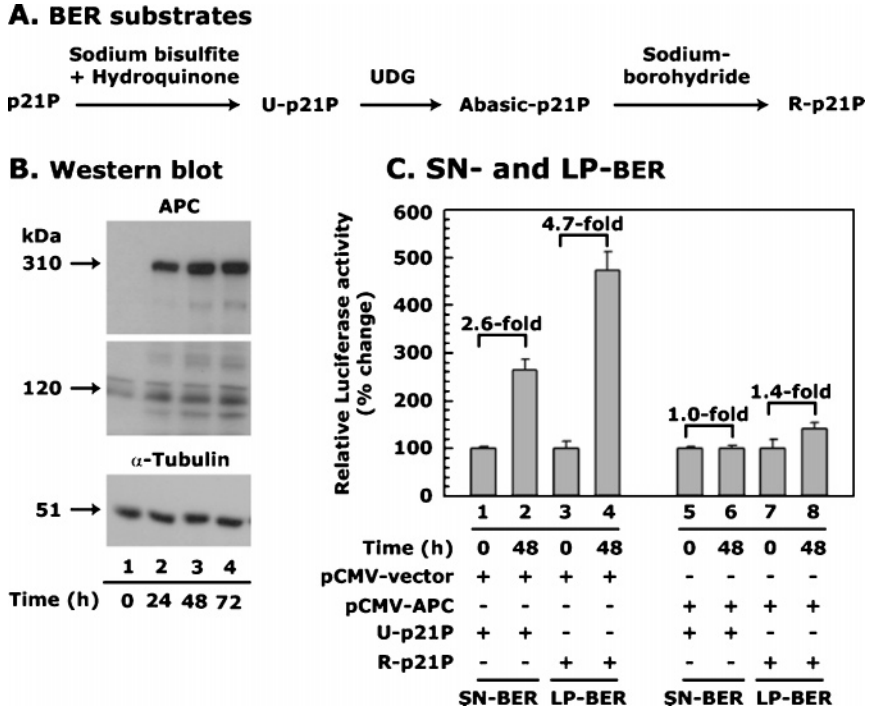

FIGURE 7: Analysis of the effect of APC on BER in LoVo cells. (A) Protocol for the modification of p21P for preparation of SN- (U-p21P) and LP-BER (R-p21P) DNA substrates. (B) Western blot analysis of the levels of overexpressed APC protein in LoVo cells. Overexpressed wild-type (310 kDa), endogenous truncated APC (120 kDa), and α-tubulin protein levels are shown. (C) Luciferase gene reporter assay of the U-p21P (lanes 1, 2, 5, and 6) and R-p21P (lanes 3, 4, 7, and 8) DNA plasmids transfected in LoVo cells. Data are the mean ± SE of three different experiments.

Overexpression of Wild-Type APC Decreases SN- and LP-BER Activities in LoVo Cells. The above experiments were performed with in vitro BER assays of purified reconstituted proteins in which a 20-amino-acid long APC peptide containing the DRI domain was used. To verify the results obtained from the in vitro reconstituted system, we used a plasmid-based BER assay system (Figure 7A), as described in our previous studies (*23*, *24*). In this assay system, upon transfection into cells, the modified p21P plasmid should show reduced promoter activity as compared to the unmodified p21P plasmid, and the promoter activity of the modified plasmid should be restored if the modified DNA is repaired by the cell. LoVo cells express a truncated APC (120 kDa), which lacks the DRI domain, does not interact with Pol-β or Fen-1 proteins, and has no effect on SN- or LP-BER activities (*23*, *25*). Thus, overexpression of wild-type APC in LoVo cells should block SN- and LP-BER activities. Wild-type APC was overexpressed through transfection with pCMV-APC overexpression plasmid. Simultaneously, U-p21P or R-p21P plasmids were also cotransfected with pCMV-APC. Overexpression of the wild-type APC protein (310 kDa) was considered optimal at the 48 h time point (Figure 7B, lane 3). Analysis of the U-p21P and R-p21P promoter activities in the absence of wild-type APC indicated an increase in the promoter activities at 48 h (Figure 7C, compare lanes 1 and 2 and lanes 3 and 4). This increase in promoter activity was reduced upon overexpression with wild-type pCMV-APC (Figure 7C, compare lanes 5 and 6 and lanes 7 and 8). From these results, we have concluded that the enhanced levels of wild-type APC blocked SN- and LP-BER in LoVo cells.

Knockdown of APC Increases SN- and LP-BER Activities in HCT-116 Cells. To recapitulate the role of APC in BER, we knocked down APC expression in HCT-116 cells by pSiRNA-APC plasmid as described in our earlier studies (*23*). Later, we established pSiRNA-APC and pSiRNA-APCmut expression cell lines: HCT-116-APC(KD) and HCT-116-APC(WT), respectively. The APC protein level was about 90% decreased in HCT-116-APC(KD) versus HCT-116-APC(WT) cells (Figure 8A). The R-p21P promoter activity in HCT-116-APC(WT) cells at the 30 h time point was 23.6-fold higher than at the 0 time point, as compared to 1.6-fold higher with U-p21P promoter when compared at the same time points (Figure 8B, compare lanes 3 and 4 and lanes 1 and 2, respectively). These results suggested that the repair of R-p21P plasmid was higher than the repair of U-p21P plasmid in HCT-116-APC(WT) cells. When these activities were compared in HCT-116-APC(KD) cells, both U-p21P and R-p21P promoter activities were about 4-fold higher than in HCT-116-APC(WT) cells (Figure 8B, compare lanes 5 and 6 and lanes 7 and 8, respectively). From these results, we have concluded that the decreased U-p21P and R-p21P promoter activities in HCT-116-APC(WT) cells were due to compromised BER activity in the presence of APC compared with APC-knockdown HCT-116-APC(KD) cells.

APC-Deficient Nuclear Extracts Are Highly Competent for BER. To further establish the role of endogenous APC in the blockage of BER, we established an in vitro BER assay with purified nuclear extracts of HCT-116-APC(WT), HCT-116-APC(KD), LS411N, and LoVo colon cancer cell lines (Figure 9A). The HCT-116 cell line expresses a 310 kDa wild-type APC, while LS411N and LoVo cell lines express 87 and 120 kDa proteins, respectively, which lack the DRI domain (*25*). We performed a LP-BER assay with these nuclear extracts. Results showed a time-dependent increase in LP-BER activity with HCT-116-APC(KD), LS411N, and

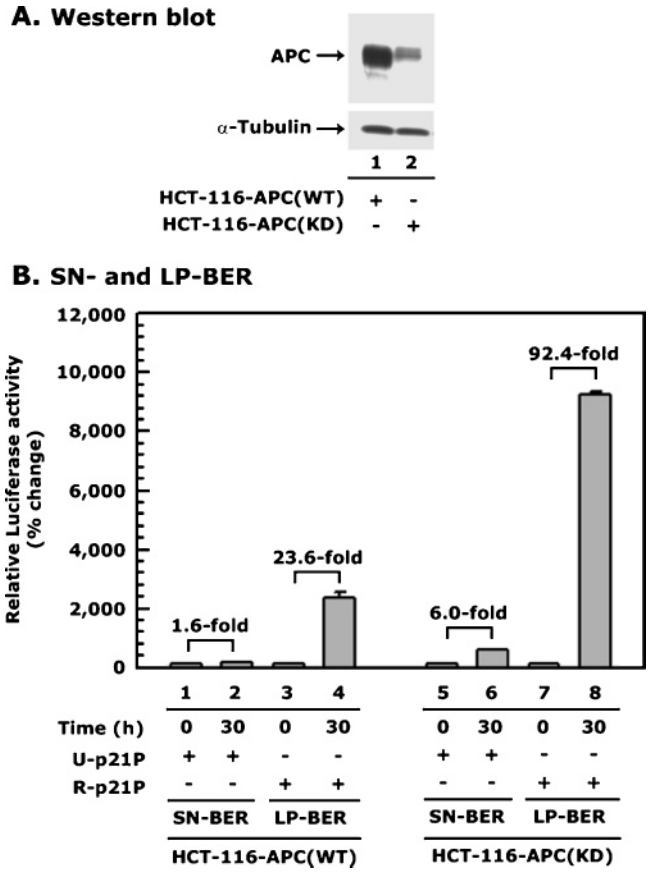

FIGURE 8: HCT-116-APC(KD) cells have higher BER capacity than HCT-116-APC(WT) cells. (A) APC protein levels in pSiRNA-APCmut (lane 1) and pSiRNA-APC stable HCT-116 cell lines (lane 2). (B) Luciferase gene reporter assay of the U-p21P and R-p21P plasmids transfected in HCT-116-APC(WT) or HCT-116-APC(KD) cell lines. Data are the mean ± SE of three different experiments.

LoVo cell nuclear extracts, which was much higher than the LP-BER activity with the HCT-116-APC(WT) nuclear extract (Figure 9B, compare lanes 2−4 with lanes 5−7, 8−10, and 11−13, respectively). Robust LP-BER was observed with LS411N and LoVo nuclear extracts when compared to the HCT-116-APC(WT) nuclear extract. LP-BER activity with HCT-116-APC(KD) nuclear extract was comparatively less than LS411N and LoVo nuclear extracts, perhaps due to the presence of residual wild-type APC level in the HCT-116-APC(KD) cells. Nonetheless, these results clearly supported our findings that the presence of APC was inhibitory to LP-BER. Similar results were obtained with SN-BER as well (data not shown).

HCT-116-APC(WT) Cells Are More Sensitive to MMS Treatment Than HCT-116-APC(KD) Cells. To better understand the biological significance of APC in the Pol-β-mediated blockage of BER, and thus cellular toxicity by the DNA alkylating agent methylmethane sulfonate (MMS), we used a clonogenic assay approach. For these experiments, we hypothesized that the toxic abasic lesions produced by MMS treatment were less repairable in the presence of APC. Therefore, they could cause severe cytotoxicity and decrease cell survival of HCT-116-APC(WT) cells in comparison with HCT-116-APC(KD) cells. The clonogenic cell survival data for HCT-116-APC(WT) and HCT-116-APC(KD) cell lines are given in Figure 10. As expected, the results showed a lower number of colonies in HCT-116-APC(WT) cells after MMS treatment than in HCT-116-APC(KD) cells (Figure 10). Thus, APC determined the cytotoxic response to MMS treatment and increased sensitivity to HCT-116-APC(WT) cells due to compromised BER activity.

DISCUSSION

Our studies have suggested that APC played a paradoxical role in blocking BER. In this investigation, we determined the mechanisms by which APC blocked BER. The BER pathway protects the genome of the cell by removing damaged nucleotides and abasic sites generated by a variety of exogenous and endogenous DNA-damaging agents (*2*). The results generated by use of in vitro reconstituted BER assay systems suggest that differential utilization of the SN- and LP-BER pathways is determined by the occurrence of regular or modified abasic sites (*46*) and/or the availability of ATP (*47*). It is apparent, however, that regulation of the SN- and LP-BER pathways is complex, particularly when the interactions of the BER system with the many cellular proteins that can modify BER activities are taken into account. For example, Werner's syndrome protein (*48*, *49*), poly(ADP-ribose)polymerase 1 (*50*, *51*), proliferating cell nuclear antigen (*3*, *38*, *52*, *53*), p53 (*54*), replication protein A (*55*), X-ray cross-complementing group 1 (XRCC1) (*56*), and arginine methyltransferase 6 (*57*) function as accessory proteins in the BER pathways. These accessory proteins interact directly with one or more of the BER proteins and alter their activity. Our studies suggest that APC is another accessory protein which interacts with Pol-β and Fen-1 and blocks strand-displacement synthesis (*23*, *25*).

This study indicates that APC interacts with a specific region of Pol-β. On the basis of the yeast-two hybrid analyses with deletion mutants of Pol-β and wild-type APC, we identified residues 80−120 of Pol-β as critical for the interaction with APC. Upon examination of the crystal structure of Pol-β and taking into account the solvent accessibility of this stretch of amino acids, we identified several residues that could interact with APC (Figure 2). Alanine substitution for Thr79, Lys81, and Arg83 abolished the interaction with APC. Notably, these amino acids are located in the linker region, which connects the lyase and polymerase domains of Pol-β (*26*).

The interaction of APC with residues Thr79, Lys81, and Arg83 of Pol-β blocked both LP- and SN-BER activities. However, the mechanism by which APC blocks LP-BER appear to differ from the mechanism by which it blocks SN-BER activities. In the LP-BER pathway, Fen-1 plays an essential role in repair. We have found that APC interacts with Fen-1 and blocks its ability to remove the 5′-endonuclease flap (*23*), thus blocking LP-BER. Removal of the 5′-ligase blocking dRP residue is a key step in both LP- and SN-BER. The single-stranded DNA-flap with the 5′-residue created from strand-displacement DNA synthesis must be removed to create the necessary 5′-phosphate required for ligation. Single-nucleotide incorporation is similar for both Pol-βWt and Pol-βMut-1 proteins. Thr79 is located between two HhH motifs and the N-subdomain of Pol-β and is important for positioning of DNA within the active site. Improper positioning of DNA within the Pol-β active site of a T79S variant of Pol-β has been postulated to modulate fidelity (*58*).

Our studies indicate that APC affects SN-BER activity by modifying the dRP-lyase activity of Pol-β. In SN-BER,

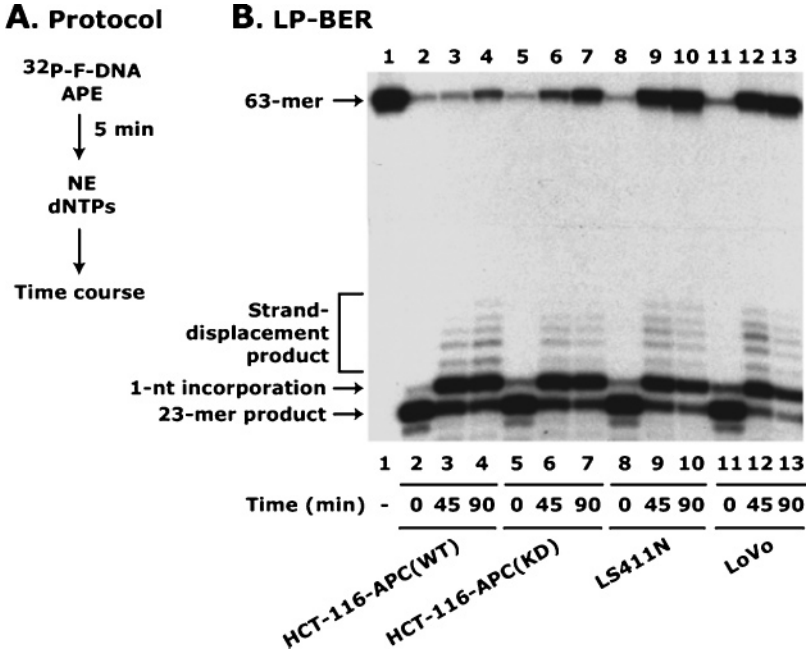

FIGURE 9: Nuclear extract with decreased APC protein level has increased F-DNA repair capacity. (A) Protocol for LP-BER with purified nuclear extracts. (B) Autoradiograph of LP-BER. The nuclear extracts from HCT-116-APC(WT) (lanes 2−4), HCT-116-APC(KD) (lanes 5−7), LS411N (lanes 8−10), and LoVo (lanes 11−13) cell lines were assembled with APE precut ^{32}P-F-DNA and dNTPs. Time-dependent repair of ^{32}P-F-DNA is shown. Lane 1, uncut 63-mer ^{32}P-labeled F-DNA. Data are representative of two independent experiments.

the dRP-lyase activity of Pol-β is the rate-limiting step (*59*). Removal of the 5′-dRP moiety is essential to generate the 5′-phosphate required for DNA ligase I to seal the nick (*26*). Previous studies trapping the Schiff base intermediate between Pol-β and the dRP-containing DNA substrate (*30*), site-directed mutagenesis (*31*), and mass spectrometry (*32*) have identified Lys72 as the Schiff base nucleophile. Interaction of APC with Pol-β occurs with a stretch of residues near Lys72 (Figure 2). The crystal structure of Pol-β indicates that Lys35, Lys68, Lys72, and Lys84 coordinate the 5′-phosphate of a gapped DNA and may modulate its dRP-lyase activity (*29*). Since the interaction of APC with Thr79, Lys81, and Arg83 falls near the dRP-lyase active-site pocket, it is not unexpected that this interaction would alter the dRP-lyase activity. Interestingly, APC inhibits SN-BER but does not block single-nucleotide incorporation for Pol-β or Pol-βMut-1. In a general understanding, it is proposed that Pol-β incorporates a single nucleotide by removing the 5′-dRP moiety in SN-BER. In our studies, Pol-βMut-1 removes the 5′-dRP moiety and displaces this residue. It appears that the single-nucleotide incorporation by Pol-β or Pol-βMut-1 occurs prior to dRP-lyase activity, which will be examined in future studies.

By combining analysis of protein−protein interactions by the yeast two-hybrid assay and the structure of Pol-β, we identified the region of Pol-β that interacts with APC and made specific amino acid substitutions that can disrupt binding to APC. The binding region links the AP-lyase domain with the polymerase domain. Amino acid changes that disrupted binding to APC also altered the catalytic activity of Pol-β and complicated analysis of the effect of APC binding on Pol-β activity. Specifically, the amino acid changes markedly reduce strand-displacement synthesis by Pol-β. This suggests that the orientation of the lyase domain with respect to the catalytic domain modulates the strand-displacement activity of Pol-β.

Mutations in the *APC* gene are one of the initiating events of colorectal carcinogenesis (*21*). It is widely accepted that APC acts as a tumor suppressor (*16*). Generally, after DNA damage, tumor suppressor genes stimulate DNA repair machinery to protect the integrity of the genome (*60*). The blockage of BER by APC could conceivably serve as a tumor suppressor function if, due to the accumulation of DNA damage, it results in apoptosis. We have previously shown that *APC* gene expression is induced in human colon cancer and in spontaneously immortalized normal human breast epithelial cell lines upon exposure to the DNA-alkylating agents *N*-methyl-*N*′-nitro-*N*-nitrosoguanine (MNNG), methylmethane sulfonate (MMS), and dimethylhyrdazine (DMH), as well as the cigarette smoke carcinogen 7,12-dimethylbenzanthracine (DMBA) (*25*, *61*−*63*). More recently, we have shown that cigarette smoke condensate induces APC levels in spontaneously immortalized normal human breast epithelial cells, blocks BER, and causes transformation of these cells (*24*, *64*). These findings point out the role of APC in carcinogenesis. On the other hand, in earlier studies we found that treatment of human colon cancer cells and mouse embryonic fibroblast cells with MMS enhanced the levels of APC and blocked BER, resulting in increased sensitivity and apoptosis of cells harboring damaged DNA (*25*, *65*). In this study, we further determined the physiological significance of the role of APC in DNA damage-induced sensitivity of HCT-116-APC(WT) versus HCT-116-APC(KD) cells lines in a clonogenic assay. We observed decreased colony formation in HCT-116-APC(WT) cells compared with HCT-116-APC(KD) cells after treatment with MMS. These results suggest that increased levels of APC after MMS treatment block BER and decrease cell growth.

In conclusion, our findings indicate that APC blocks LP-BER by blocking Fen-1 activity and SN-BER by blocking the dRP-lyase activity of Pol-β. Our results also suggest that the linker region of Pol-β plays a functional role during

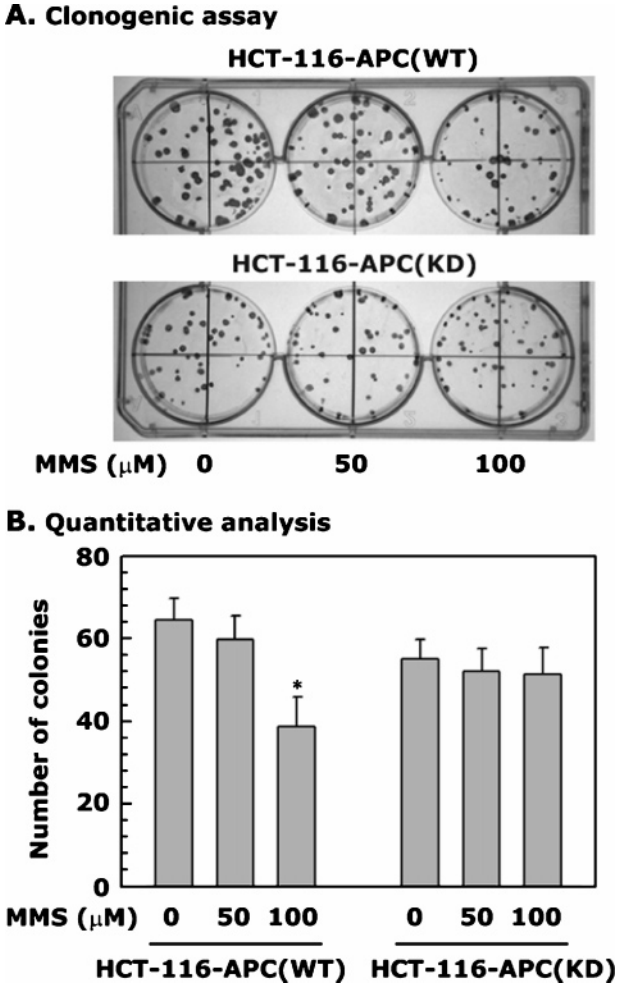

FIGURE 10: MMS-induced loss of clonogenicity in HCT-116-APC-(WT) cells. Both HCT-116-APC(WT) and HCT-116-APC(KD) cell lines were treated with different concentrations of MMS. After 72 h, the medium was replaced and cells were grown for an additional 8 days. Cells were stained with 0.025% crystal violet and colonies were counted. (A) Photograph of the clonogenic assay plates. (B) Graphical representation of the data, which are the mean ± SE of three different experiments.

strand-displacement DNA synthesis. Since strand-displacement DNA synthesis is mediated by Fen-1, APC blocks strand-displacement synthesis of both F-DNA and U-DNA substrates by inhibiting Fen-1 activity. Furthermore, since the DNA repair inhibitory (DRI) domain is retained in most APC proteins that are mutated in the mutator cluster region (MCR) (*21*), both the wild-type and mutant APC (retaining the DRI domain) proteins can interact with Pol-β and Fen-1 and can block SN- and LP-BER activities. Our assays of SN- and LP-BER in the in vitro reconstituted system, live cell system, and nuclear extract system all suggest that the levels of APC are critical for the repair of the BER pathways in colon cancer cells. More detailed knowledge of the mechanisms that determine the carcinogenic and cytotoxic effects of the blockage of BER by APC on normal cells and tumor cells is required for successful development of therapeutic strategies based on inhibitors of this interaction. Nonetheless, our studies provide important, new information and understanding about the in vivo regulation of BER. Our finding adds to the recent evidence that, contrary to the prevalent dogma about the straightforward nature of BER involving only a few enzymes, many ancillary proteins play a critical role in the complex regulation of the BER process in vivo. These proteins, including APC, may be involved in specific subpathways of BER that could be triggered by unknown signaling. Characterization of the subpathways and of the parameters that control them in vivo poses a major challenge.

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SUPPORTING INFORMATION AVAILABLE

Time and concentration dependence of strand-displacement synthesis with Pol-βWt and Pol-βMut-1 proteins (Figures S1 and S2), and LP-BER activity of Pol-βWt and Pol-βMut-1 proteins with ^{32}P-F-DNA (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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