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Tumor Suppressor APC Blocks DNA Polymerase β-dependent Strand Displacement Synthesis during Long Patch but Not Short Patch Base Excision Repair and Increases Sensitivity to Methylmethane Sulfonate*

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In the present investigation, we report a previously unsuspected function of the tumor suppressor protein, APC (adenomatous polyposis coli), in the regulation of base excision repair (BER). We identified a proliferating cell nuclear antigen-interacting protein-like box sequence in APC that binds DNA polymerase β and blocks DNA polymerase β-mediated strand-displacement synthesis in long patch BER without affecting short patch BER. We further showed that the colon cancer cell line expressing the wild-type *APC* gene was more sensitive to a DNA-methylating agent due to decreased DNA repair by long patch BER than the cell line expressing the mutant *APC* gene lacking the proliferating cell nuclear antigen-interacting protein-like box. Experiments based on RNA interference showed that the wild-type *APC* gene expression is required for DNA methylation-induced sensitivity of colon cancer cells. Thus, APC may play a critical role in determining utilization of long *versus* short patch BER pathways and affect the susceptibility of colon cancer cells to carcinogenic and chemotherapeutic agents.

Organisms continually repair damaged DNA. Knowledge of these repair responses is critical to our understanding of how and why the genome is affected during the lifespan of the organism. DNA repair systems efficiently remove damaged DNA via several different pathways that reverse the vast majority of genetic lesions formed during the lifespan of a cell (reviewed in Ref. 1). The base excision repair (BER)[1] system of both prokaryotes and eukaryotes is directed toward the repair of modified bases and strand breaks. It is mediated through two pathways that are differentiated by the size of the repair gap and the enzymes involved (2, 3). These pathways are designated "single nucleotide BER," also referred to as "short patch (SP) BER," and "multinucleotide (2–13-nucleotide repair patch) BER," also referred to as "long patch (LP) BER." In both pathways, repair is initiated by the removal of a damaged base by a DNA glycosylase, leaving an abasic or apurinic/apyrimidinic (AP) site. Several enzymes, acting in an orderly fashion, participate in DNA synthesis and the filling of the gaps, including *N*-glycosylase, AP endonuclease (APE), pol-β (also has deoxyribose phosphatase activity), or polymerase δ/ε, flap endonuclease 1 (Fen-1), and DNA ligase I (reviewed in Ref. 4). PCNA does not participate directly but is an important cofactor in several of these activities and has been shown to bind APE, Fen-1, and DNA ligase I through the PCNA-interacting protein-like box (PIP-like box) motifs in these proteins and to modulate their activity (reviewed in Refs. 5 and 6).

The physiologic basis for the selection of one BER pathway over the other has not been established. It has been suggested that the utilization of the SP- and LP-BER pathways is determined by the type of damage encountered on DNA (reviewed in Ref. 7). In this scenario, simple basic sites would generally be repaired through the SP-BER pathway; upon oxidation or reduction of the sites, they would be repaired through the LP-BER pathway. Recently, it has been reported that a decrease in the ATP concentration shifts utilization of the SP-BER pathway to the LP-BER pathway through the stimulation of strand displacement synthesis by pol-β (8). We have observed an increase in *APC* (adenomatous polyposis coli) gene expression upon exposure of colon cancer cells to DNA-damaging agents (9, 10), suggesting the possibility of an interaction between APC and the DNA repair machinery. Here, we provide evidence that APC plays a role in regulating DNA repair by interacting directly with pol-β and blocking its ability to mediate LP-BER strand displacement synthesis. The blocked LP-BER then increases sensitivity of colon cancer cells to DNA-methylating agent, which in turn causes DNA damage that is repaired by LP-BER.

MATERIALS AND METHODS

Preparation of Nuclear Extract—Nuclear extracts were prepared as described by Shapiro *et al.* (11) from HCT-116, LoVo, and LS411N colon cancer cell lines as well as from the isogenic mouse embryonic fibroblast (MEF) cell line that contain either the wild-type (MEF-polβ) or a cell line in which the DNA polymerase gene β (*pol-β*) has been knocked out (MEF-polβKO).

APC Peptides—A peptide, 20 amino acids in length (KVSSINQETIQTYCVEDTPI), representing the PIP-like box of the wild-type APC, APCwt-(1250–1269), or a mutated form of the APC, APCmut-(1250–1269), in which amino acids 1256, 1259, and 1262 were replaced with alanine (KVSSINAETAQTACVEDTPI) was synthesized at the Protein Chemistry and Biomarkers core facility of the ICBR of the University of Florida.

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[1] The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; APE, apurinic/apyrimidinic endonuclease; pol-β, DNA polymerase β; F, 3-hydroxy-2-hydroxymethyltetrahydrofuran; Fen-1, flap endonuclease 1; LP, long patch; MMS, methylmethane sulfonate; PCNA, proliferating cell nuclear antigen; PIP, PCNA-interacting protein; SP, short patch; U, uracil; UDG, uracil-DNA glycosylase; MEF, mouse embryo fibroblast; BD, binding domain; AD, activation domain; MMR, mismatch repair; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Pull-down Assays—For pull-down assays, 150–200 μg of nuclear extracts were precleared by incubating them with 2 μg of rabbit IgG at 4 °C for 2 h. The mixture was rocked for 2 h, a slurry of bovine serum albumin-blocked protein A-Sepharose CL-4B beads was added, and the mixture was incubated for an additional 2 h at 4 °C. The beads were removed by centrifugation, and the precleared supernatant was incubated with anti-APC rabbit polyclonal antibody (Bio-Synthesis Inc., Lewisville, TX) at 4 °C for 4 h. Protein A-Sepharose CL-4B beads were then added, and the mixture was incubated for a further 3–4 h at 4 °C and then washed five times with a washing buffer (pH 7.9) containing 20 mM Hepes buffer, 100 mM KCl, 5% (v/v) glycerol, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.05% (v/v) Nonidet P-40 to remove unbound proteins.

Western Blot Analysis—To determine the interaction of APC with PCNA and pol-β, Western blot analysis of the immunocomplexes was carried out as described previously (9). The antibodies used were procured as follows: anti-human pol-β (mouse monoclonal) (12); anti-PCNA (mouse monoclonal) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-APC (Ab-1) from Oncogene Research Products (Cambridge, MA), and anti-α-tubulin from Sigma.

Far Western Blot Analysis—APCwt-(1250–1269) and APCmut-(1250–1269) peptides and PCNA and glutathione *S*-transferase-p21 proteins were slot-blotted (0–10 μg) onto a polyvinylidene difluoride membrane (Amersham Biosciences) in a binding buffer containing 20 mM Tris-Cl, pH 7.4, 100 mM phosphate buffer, pH 7.4, 60 mM KCl, 0.25% Nonidet P-40. After blotting, the membrane was blocked with 5% bovine serum albumin and washed three times with Tris-buffered saline with 0.025% (v/v) Tween 20 prior to incubation with purified PCNA or pol-β proteins. Binding was detected using anti-PCNA or anti-pol-β antibodies as the primary antibodies and horseradish peroxidase-conjugated goat-anti-mouse IgG as the secondary antibody. The signals were detected using the enhanced chemiluminescence technique (Amersham Biosciences).

Yeast Two-hybrid Analysis—The yeast two-hybrid system was used to determine the functional interaction of APC with pol-β *in vivo*. The *APC* cDNA fragments containing the wild type (amino acids 1190–1328) or mutant (amino acids 1200–1324) PIP-like box binding motifs for protein-protein interaction studies were fused to the yeast GAL4 DNA binding domain (BD) in plasmid pGBDU-C3. The interacting proteins such as full-length pol-β and PCNA were fused with the yeast GAL4 activation domain (AD) in plasmid pGAD-C3 (13). Adapters were included as needed for in-frame insertion of APC, pol-β, and PCNA sequences relative to GAL4 BD or GAL4 AD plasmids. The yeast strain *S. cerevisiae* PJ69–4A was co-transformed with pGBDU-C3- and pGAD-C3-derived plasmids (13). After transformation, cells were spread on plates containing yeast synthetic dropout (SD)-UL medium lacking only vector markers Ura for pGBDU-C3-derived plasmids and Leu for pGAD-C3-derived plasmids. To test potential protein-protein interactions, transformed cells were screened for growth on yeast SD-ULH medium (SD medium lacking Ura, Leu, and His), but containing 5 mM His3 inhibitor, 3-amino-1,2,4-trizole, to prevent *His3* reporter gene auto-activation. Protein interactions were further confirmed by a β-galactosidase filter lift assay.

Synthesis and Labeling of DNA Substrates—The nucleotide sequence of the 63-mer oligonucleotide for SP-BER has a U residue at position 24 and is referred as U-DNA (5′-TAGATGCCTGCAGCTGATGCGCUG-TACGGATCCACGTGTACGGTACCGAGGGCGGGTCGACA-3′). To examine LP-BER activity, the 63-mer AP site analog (3-hydroxy-2-hydroxymethyltetrahydrofuran) was synthesized as described earlier and is referred to as F-DNA (14). The nucleotide sequence of F-DNA is the same as for U-DNA, except that the U has been replaced with F. After synthesis, these DNA substrates were gel-purified, labeled with [γ-^{32}P]ATP at the 5′-end using T-4 polynucleotide kinase, and annealed with a complementary oligonucleotide with a G residue opposite to the AP site.

In Vitro BER Assay—The BER reaction was reconstituted using purified proteins under the following conditions. The reaction mixture for LP BER contained 30 mM Hepes, pH 7.5, 30 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 0.01% Nonidet P-40, 0.5 mM ATP, and 10 μM each of dATP, dCTP, dGTP, dTTP in a final volume of 20 μl. The BER reaction mixture was assembled on ice by the addition of 0.5 nM APE, 10 nM PCNA, 2.5 nM pol-β, 10 nM Fen-1, and 100 nM DNA ligase I and incubated for 5 min. The concentrations of the APCwt-(1250–1269) and APCmut-(1250–1269) peptides used in each experiment are given in the figure legends. For SP-BER, the reaction mixture was the same except that 1 unit of uracil-DNA glycosylase (UDG) was added at the beginning of the incubation. The reactions were initiated by the addition of 2.5 ng of ^{32}P-labeled F- or U-DNA substrates for LP- and SP-BER, respectively. The reaction mixture was then incubated for 30 min at 37 °C. The reaction was terminated by the addition of 0.4% (w/v) SDS, 5 mM EDTA, 1 μg of proteinase K and 10 μg of carrier RNA. After incubation for an additional 30 min at 37 °C, the DNA was recovered by phenol/chloroform extraction and ethanol precipitation.

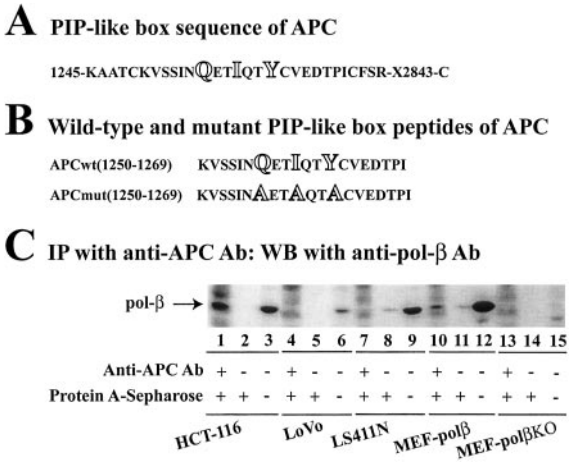

FIG. 1. **Pull-down analysis of the interaction of APC and pol-β.** *A*, the PIP-like box motif. The *number* to the *left* of the amino acid indicates its position in the whole sequence. The most conserved amino acid residues are marked with *hollow letters*. *B*, the sequence of the peptides representing the APC wild-type PIP-like box and the APC mutated PIP-like box, in which the consensus residues of the PIP-like box have been replaced with alanine. *C*, an immunoblot of pol-β. The APC protein complexes from HCT-116, LoVo, LS411N, MEF-polβ, and MEF-polβKO cell nuclear extracts (150 μg) were immunoprecipitated with anti-APC antibody and then immunoblotted with anti-pol-β antibody. A positive control with one-seventh of the nuclear extract used for immunoprecipitation is shown in *lanes 3*, *6*, *9*, *12*, and *15*.

APC Gene Silencing Assays—For *APC* gene silencing, the small interfering RNA sense and antisense oligonucleotides 5′-GATCCGCAA-CAGAAGCAGAGAGGTTTCAAGAGAACCTCTCTGCTTCTGTTGCTT-TTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAAGCAACAGAAGCAGA-GAGGTTCTCTTGAAACCTCTCTGCTTCTGTTGCG-3′, respectively, were annealed and subcloned into pSilencer 2.1 vector system (Ambion Inc., Austin, TX) to generate pSiRNA-APC plasmid (15). A 19-nucleotide sequence of the pSiRNA-APC plasmid was scrambled to generate pSiRNA-APCmut plasmid. The sense and antisense nucleotide sequences for the pSiRNA-APCmut plasmid were 5′-GATCCGGACAGT-AAGGAAGAACGCTTCAAGAGAGCGTTCTTCCTTACTGTCCTTTTT-TGGAAA-3′ and 5′-AGCTTTTCCAAAAAAGGACAGTAAGGAAGAAC-GCTCTCTTGAAGCGTTCTTCCTTACTGTCCG-3′, respectively. Specificity of the mutant 19-nt sequence was confirmed by a BLAST search against the human genome sequence.

The isogenic HCT-116, HCT-116+Ch3, and HCT-116+Ch2 human colon cancer cell lines were grown in 96-well tissue culture plates in McCoy's 5A medium containing 10% (v/v) of fetal bovine serum, except 400 μg/ml of G418 sulfate was added in the medium of HCT-116+Ch3 and HCT-116+Ch2 cell lines. Cells were transfected with the above plasmids using Lipofectamine (Invitrogen). After 16 h of transfection, the cells were starved for 16 h and then treated with different concentrations of DNA-methylating agent, methylmethane sulfonate (MMS; Aldrich). After 1 h of treatment, the MMS-containing medium was replaced with fresh medium containing 10% (v/v) fetal bovine serum. The survival of the cells was measured after 24 h by MTT assay (ATCC, Manassas, VA). Parallel experiments were performed for preparing cell lysates to measure the APC protein levels in treated and untreated cells (9).

Comet Assay—For evaluating DNA damage and repair, a single cell gel electrophoresis (comet assay) kit was used (Trivigen, Gaithersburg, MD). The HCT-116 cells were transfected with pSiRNA-APC or pSiRNA-APCmut plasmids and treated with MMS for 1 h. After treatment, the medium was replaced with a fresh medium containing 10% (v/v) fetal bovine serum and incubated for an additional 24 h. Plasmid transfection and MMS treatment conditions to the cells were the same as described above. The single-cell gel electrophoresis of DNA was performed as described by the manufacturer. DNA from cells was

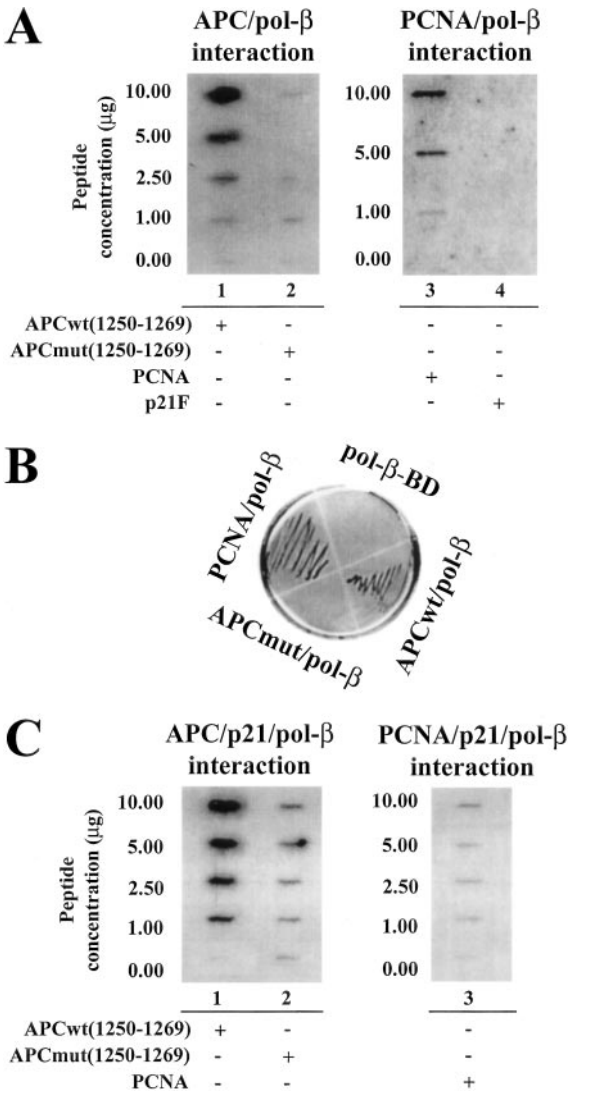

FIG. 2. **Far Western blot analysis of the interaction of APC and pol-β.** *A*, a far Western blot analysis of APC/pol-β interaction. The interaction of PCNA and pol-β is also shown as a positive control. *p21F*, amino acids 1–164. *B*, interaction of APC and pol-β by yeast two-hybrid analysis. The yeast two-hybrid constructs are described under "Materials and Methods." The yeast PJ69–4A cells were co-transformed with pGBDU-C3-APCwt or pGBDU-C3-APCmut plasmids with pGAD-C3-pol-β plasmid. For a positive control, PCNA and pol-β interaction is shown. Transformation with pol-β-BD alone served as a negative control for background colonies. *C*, far Western blot analysis to determine specificity of the interaction of APC with pol-β. *p21F*, full-length p21 (amino acids 1–164).

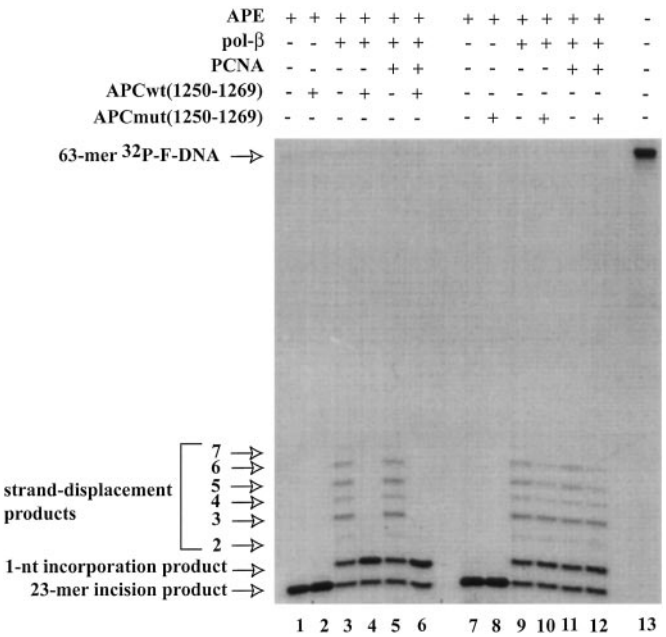

FIG. 3. **Pol-β-dependent blockage of strand displacement synthesis in LP-BER by APCwt-(1250–1269) peptide.** The APE, pol-β, PCNA, and 2 μM of APCwt-(1250–1269) or APCmut-(1250–1269) peptide were mixed and preincubated for 5 min on ice. The repair reaction was initiated with [^{32}P]F-DNA and dNTPs and incubated for 30 min at 37 °C. The *arrows* show the different BER products, including the 23-mer incision, 1-nt incorporation product, and 2–7-nt strand displacement products. *Lane 13* shows the position of the 63-mer [^{32}P]F-DNA. *nt*, nucleotide.

visualized using SYBRgreen dye. Images were captured using a fluorescence microscope (Zeiss Axioplan-2 Imaging, Thornwood, NY) at ×20 magnification.

RESULTS

APC Has PCNA- and Pol-β-binding Motifs—After observing an increase in *APC* gene expression in response to DNA-damaging agents (9, 10), we used a bioinformatics approach to explore the possibility that APC expresses binding sites for known components of the two pathways of BER. The presence of a PIP-like box in APC would be of particular interest, since this well conserved motif mediates interactions of PCNA with other DNA repair/replication proteins (reviewed in Refs. 5 and 6) including p21(Waf-1/Cip1), xeroderma pigmentosum group G, DNA-(cytosine-5)-methyltransferase, replication factor C-p140, polymerase δ third subunit, MSH6, uracil-DNA-glycosylase 2, and a mammalian ortholog of the *Escherichia coli* MutY DNA glycosylase (MYH) as well as APE, pol-β, Fen-1, and DNA ligase I (16) (reviewed in Refs. 5 and 6). The PIP-like box consists of a sequence Q*XX*(h)*XX*(a)(a), in which "h" represents amino acid residues with moderately hydrophobic side chains (*e.g.* Leu, Ile, or Met), "a" represents residues with highly hydrophobic side chains (*e.g.* Phe and Tyr), and "*X*" is any residue (reviewed in Ref. 5). A matrix for the PIP-like box was built using the meta-MEME program developed by Bailey and Elkman (17), which was used for a search of the motif using the program as described by Zhou *et al.* (18). The meta-MEME program identified a conserved PIP-like box between residues 1245 and 1273 of APC (Fig. 1*A*).

In extending these studies to explore the possibility of whether DNA repair proteins interact with APC, an interaction with pol-β was discovered. Pull-down assays showed that wild-type APC physically associates with pol-β in nuclear extracts of HCT-116 cells as well as mouse embryonic fibroblast cells that express wild-type pol-β (12) (Fig. 1*C*, *lanes 1–3* and *10–12*, respectively) but not in nuclear extracts of LoVo and LS411N cells, which lack the PIP-like box in APC (Fig. 1*C*, *lanes 4–6* and *7–9*, respectively), or MEF-polβKO cells (12), which lack pol-β (Fig. 1*C*, *lanes 13–15*). This interaction was further confirmed by far Western analysis, in which the wild-type APC peptide containing the PIP-like box, APCwt-(1250–1269), was found to bind to pol-β in a dose-dependent manner, whereas mutant peptide, APCmut-(1250–1269), in which the critical residues in the PIP-like box motif were replaced with alanine (Fig. 1*B*), did not (Fig. 2*A*, *lanes 1* and *2*, respectively). The further pull-down experiments showed an interaction of APC with PCNA but no interaction with other BER proteins such as APE and DNA ligase I (data not shown).

The above *in vitro* interactions of APC and pol-β were further confirmed by yeast two-hybrid analysis. A positive interaction was observed between APCwt and pol-β but not with APCmut-expressing plasmids (Fig. 2*B*). The plasmid that contained only

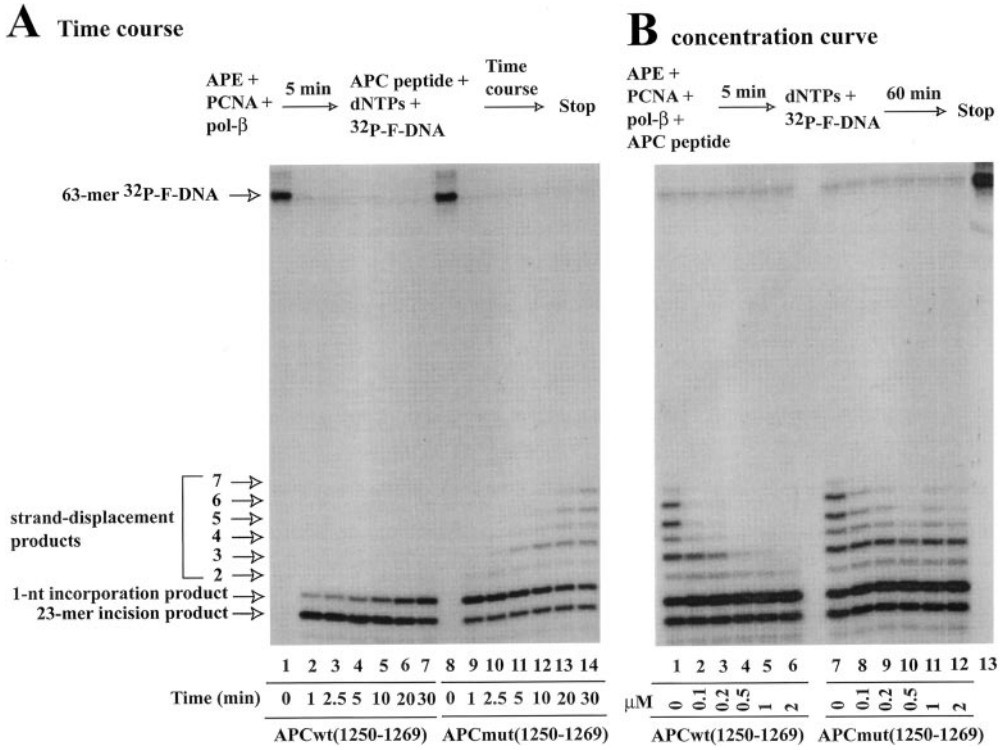

FIG. 4. **Characterization of the effect of APC peptide on strand displacement synthesis.** *A*, time course. The BER reaction mixture was assembled with APE, PCNA, and pol-β for 5 min on ice. Then 2 μM of APCwt-(1250–1269) or APCmut-(1250–1269) peptide, [^{32}P]F-DNA, and dNTPs were added and incubated at 37 °C. At different time intervals, an aliquot of the reaction mixture was removed. *B*, concentration curve. The BER reaction mixture was assembled with APE, PCNA, pol-β, and different concentrations of APCwt-(1250–1269) or APCmut-(1250–1269) peptide for 5 min on ice. Then [^{32}P]F-DNA and dNTPs were added and incubated for 30 min at 37 °C. The reaction products were resolved by electrophoresis. *Lane 13* shows the position of the 63-mer [^{32}P]F-DNA. The *arrows* show different BER products including a 23-mer incision product, 1-nt incorporation product, and 2–7-nt strand displacement products.

pol-β-binding domain (pol-β-BD) served as a negative control for background colonies. To verify our assay system, we used PCNA- and pol-β-expressing plasmids (Fig. 2*B*), whose interaction has been shown in earlier studies (16). These results support that APC interacts with pol-β both *in vitro* and *in vivo* conditions.

The finding that the PCNA and pol-β can interact with APC at the same PIP-like box region was surprising. Peptides with PCNA-binding motifs such as p21 bind to specific sites on the interdomain connector loops of PCNA (19, 20). If pol-β has a binding site on its surface that is like those on PCNA that would accommodate binding by a PCNA-binding motif, then other proteins such as p21 with a PIP box should compete APC for binding to pol-β. To address this hypothesis, we performed a far Western analysis in which the pol-β binding to APC was allowed to compete with p21. The APC blot was first incubated with p21 and then with pol-β. After incubation, the blot was probed with anti-pol-β antibody to determine the interaction of p21 and pol-β with APC. Under the experimental conditions, it was expected that if p21 binds with APC then it will compete for the binding with pol-β. However, we observed a strong pol-β interaction signal with the APCwt-(1250–1269) peptide, which increased in a dose-dependent manner, and a faint signal with APCmut-(1250–1269) peptide (Fig. 2*C*, compare *lane 1* with *lane 2*). These results suggest that p21 did not compete pol-β for the binding to APC. On the other hand, when PCNA was immobilized on the membrane and incubated with p21 first and then with pol-β, the p21 completely blocked the binding of pol-β with PCNA (Fig. 2*C*, *lane 3*), suggesting a strong binding of p21 with PCNA. From these results, it can be concluded that the interaction of pol-β with APC is unique, and PCNA and pol-β do not share a common site for interaction with the PCNA-binding motif. Thus, the PIP-like box of APC may be interacting with pol-β and PCNA in different ways.

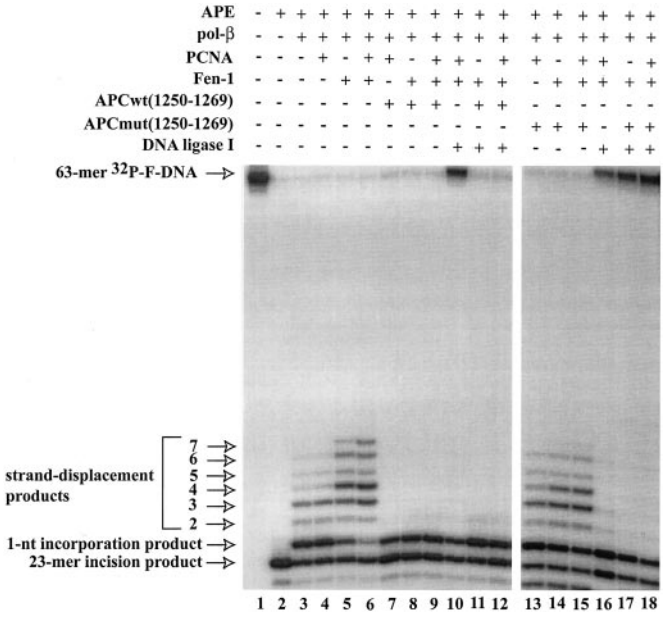

FIG. 5. **Interaction of the APCwt-(1250–1269) peptide with the LP-BER pathway occurs prior to DNA ligase ligation.** In this experiment, the BER reaction mixture was assembled on ice for 5 min with APE, pol-β, PCNA, and Fen-1 proteins and 2 μM of APCwt-(1250–1269) or APCmut-(1250–1269) peptide. The repair reaction was initiated with [^{32}P]F-DNA and dNTPs and incubated for 30 min at 37 °C. DNA ligase I was added into the reaction mixture, and incubation was continued for an additional 30 min at 37 °C.

APC Blocks Pol-β-dependent LP Strand Displacement Synthesis—Pol-β functions as a critical polymerase in BER,

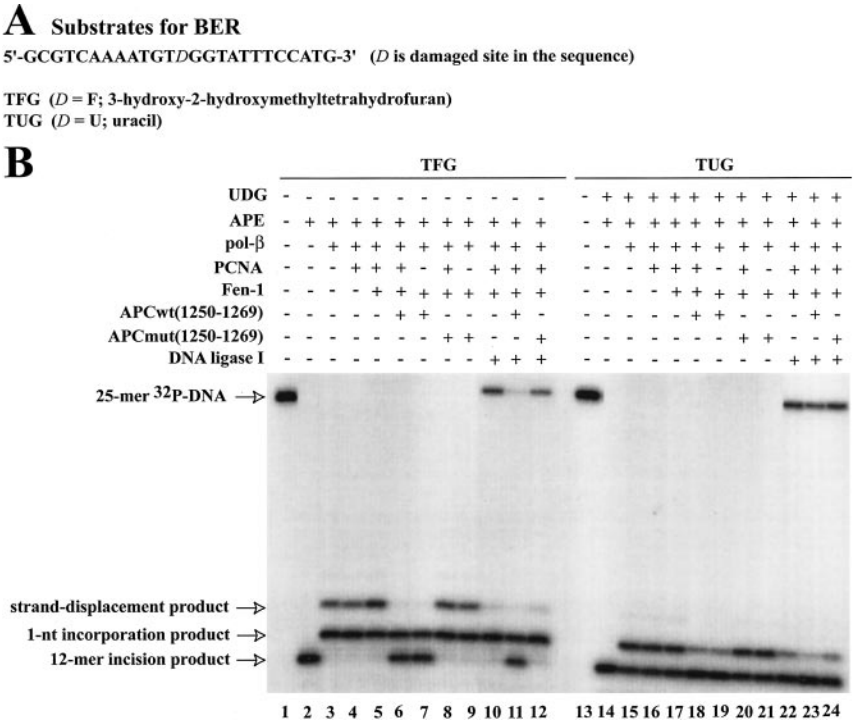

FIG. 6. The APCwt-(1250–1269) peptide blocks the LP- but not the SP-BER, as tested with 25-mer-long DNA substrates (*A*). *B*, the BER reaction mixture was assembled on ice for 5 min for short patch BER with UDG, APE, pol-β, PCNA, and Fen-1 proteins and 2 μM APCwt-(1250–1269) or APCmut-(1250–1269) peptide. For the long patch BER, the reaction mixture was the same as above, except the UDG was eliminated. The repair reaction was initiated with [^{32}P]U-DNA or [^{32}P]F-DNA for short and long patch, respectively, and dNTPs. The reaction mixture was incubated for 30 min at 37 °C. Then 100 nM DNA ligase I was added in to the reaction mixture, and incubation was continued for an additional 30 min at 37 °C.

whereas PCNA modulates the activity of pol-β (16) (reviewed in Ref. 4), APE (21), Fen-1 (22), and DNA ligase I (23) through interaction with the PIP-like box motifs in these molecules. Thus, the binding of PCNA and pol-β to the PIP-like box expressed on the APC molecule could have functional implications. The potential effects of the interactions were determined using an *in vitro* reconstitution assay, in which synthetic APC peptides were incubated for 5 min with purified APE, pol-β, and PCNA prior to the addition of dNTPs and the substrate. For analysis of LP-BER, 63-mer [^{32}P]F-DNA is used as the substrate (14). In F-DNA, the nucleotide at position 24 is replaced with 3-hydroxy-2-hydroxymethyltetrahydrofuran (F). These assays revealed that the addition of the APCwt-(1250–1269) synthetic peptide (Fig. 3, compare *lane 3* with *lane 4*) effectively blocked pol-β-mediated strand displacement without affecting pol-β-mediated 1-nucleotide incorporation (Fig. 3, *lanes 4*, *6*, and *9–12*) or APE-mediated generation of the 23-mer incision product of [^{32}P]F-DNA (Fig. 3, compare *lane 1* with *lane 2*). The addition of the APCmut-(1250–1269) peptide had no effect on any of these activities (Fig. 3, compare *lane 9* with *lane 10*). Further characterization of the effect of the APCwt-(1250–1269) peptide confirmed these results. The time course and concentration dependence of the APC-mediated block of pol-β-mediated strand displacement synthesis have been established (Fig. 4, *A* and *B*, respectively).

Although PCNA can play a role in the regulation of LP-BER (reviewed in Ref. 4), PCNA neither stimulated pol-β-mediated strand displacement synthesis (Fig. 3, compare *lanes 3* and *5*) nor interfered with the APCwt-(1250–1269)-dependent block in pol-β-mediated strand displacement synthesis (Fig. 3, compare *lanes 4* and *6*) in these assays. Since the APCwt-(1250–1269) peptide blocks pol-β-mediated strand displacement synthesis without affecting 1-nucleotide incorporation, the possibility arose that other mediators of strand displacement synthesis, such as Fen-1 and DNA ligase I, could readily compensate for the APC-induced block in pol-β activity. The APCwt-(1250–1269)-mediated block of strand displacement synthesis was not relieved by the addition of Fen-1 (Fig. 5, compare *lanes 5* and *6* with *lanes 8* and *9*, and compare *lane 13* with *lanes 14* and *15*, respectively). Moreover, the strand displacement synthesis stimulated by Fen-1 in the absence of APC was unaffected by the addition of PCNA (Fig. 5, compare *lane 3* with *lane 5* and *lane 4* with *lane 6*). The addition of DNA ligase I into reaction mixtures containing the APCwt-(1250–1269) peptide did not relieve the block in the generation of a 63-mer ^{32}P-labeled F-DNA ligated product (Fig. 5, compare *lane 10* with *lanes 11* and *12*). To eliminate the possibility that the length of the DNA affects the functional outcome, we also used 25-mer [^{32}P]F-DNA as a substrate. Similar results were

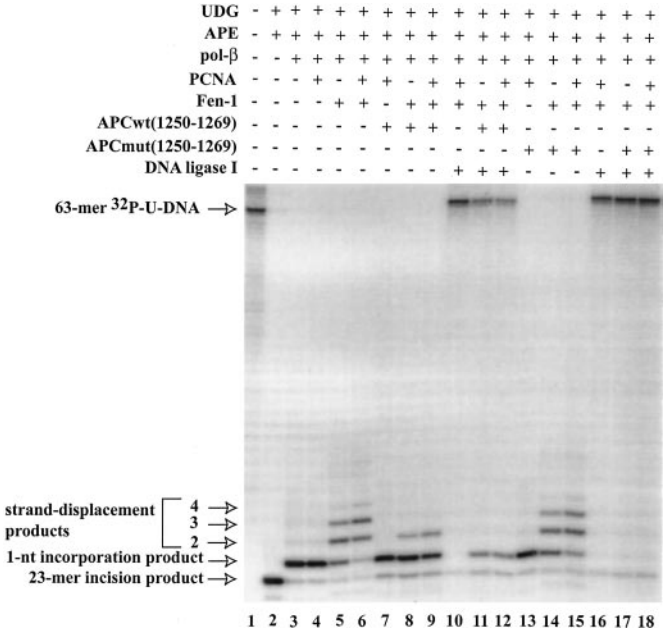

FIG. 7. **The APCwt-(1250–1269) peptide does not block SP-BER.** The BER reaction mixture was assembled on ice for 5 min with UDG, APE, pol-β, PCNA, Fen-1 proteins, and 2 μM of APCwt-(1250–1269) or APCmut-(1250–1269) peptide. After preincubation, the repair reaction was initiated with [^{32}P]U-DNA and dNTPs and incubated for 30 min at 37 °C. Then 100 nM of DNA ligase I was added to the reaction mixture, and incubation was continued for an additional 30 min at 37 °C.

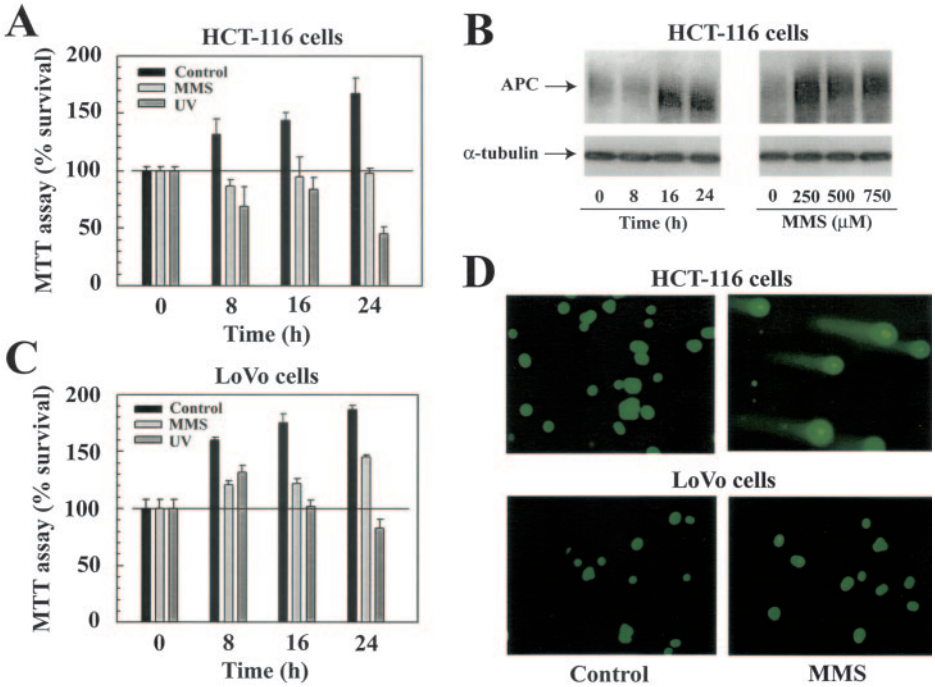

FIG. 8. **Sensitivity of colon cancer cells to MMS.** *A*, HCT-116 cells (carrying the wild-type *APC* gene) were grown in McCoy's 5A medium containing 0.5% (v/v) fetal bovine serum for 16 h and then treated with 500 μM MMS for 1 h. Another set of cells was exposed with 50 J/m^2 UV irradiation. After the treatment, the medium was replaced with a fresh medium containing 10% (v/v) fetal bovine serum. At different time intervals, cell survival was determined by MTT assay. *B*, the time and concentration curve of APC protein levels in HCT-116 cells treated with MMS. *Lower panel*, α-tubulin level that served as loading control of the proteins. *C*, the survival curve of LoVo cells (carrying mutant *APC* gene that lacks the PIP-like box) treated either with 500 μM MMS for 1 h or 50 J/m^2 UV irradiation. After the treatment, the medium was replaced with a fresh medium containing 10% (v/v) fetal bovine serum as described in *A*. *D*, comet assay. HCT-116 and LoVo cells were treated with 500 μM MMS for 1 h and then processed for the comet assay after 24 h as described under "Materials and Methods."

observed with the APCwt-(1250–1269) peptide in which pol-β-mediated strand displacement synthesis was blocked without affecting 1-nucleotide incorporation activity (Fig. 6, compare *lane 5* with *lane 6*). The APCwt-(1250–1269) peptide-mediated blockage of the pol-β-mediated strand displacement synthesis was not relieved after the addition of DNA ligase I (Fig. 6, compare *lane 10* with *lane 11*). The APCmut-(1250–1269) peptide, on the other hand, did not block the strand displacement synthesis and showed a 25-mer repaired product in the presence of DNA ligase I (Fig. 6, compare *lane 5* with *lane 8* and *lane 10* with *lane 12*, respectively).

APC Has No Effect on Pol-β-mediated SP-BER—The effects of APC on SP-BER were analyzed using 63-mer [^{32}P]U-DNA as the substrate (14) (Fig. 7). The APCmut-(1250–1269) peptide affected neither strand displacement synthesis nor DNA ligation activity during SP-BER mediated by UDG, APE, pol-β, and PCNA (Fig. 7, *lanes 13–18*). Although the repair of U-DNA is accomplished predominantly by SP-BER, we did find that the presence of Fen-1 enabled pol-β-mediated strand displacement synthesis of up to 4 nucleotides (Fig. 7, compare *lanes 3* and *4* with *lanes 5* and *6*). In contrast to the results of the analysis of LP-BER, in which 63-mer [^{32}P]F-DNA was used as the substrate, this strand displacement synthesis of 63-mer [^{32}P]U-DNA in the presence of Fen-1 was partially blocked by the APCwt-(1250–1269) peptide (Fig. 7, compare *lane 7* with *lanes 8* and *9*), suggesting that APC may have an additional effect on Fen-1 activity during the repair of U-DNA. When DNA ligase I was added into the reaction mixture, complete repair of 63-mer [^{32}P]U-DNA was accomplished (Fig. 7, *lanes 10–12*). Upon utilization of 25-mer [^{32}P]U-DNA as the substrate, however, strand displacement synthesis did not occur even in the presence of Fen-1 (Fig. 6, compare *lane 16* with *lane 17*). This implies that the length of the DNA substrate is critical to Fen-1-supported stimulation of pol-β-mediated strand displacement synthesis. In the presence of DNA ligase I, complete repair of the 25-mer [^{32}P]U-DNA was observed in the presence of the APCwt-(1250–1269) peptide (Fig. 6, compare *lane 22* with *lane 23*), which blocked the repair of 25-mer [^{32}P]F-DNA (Fig. 6, compare *lane 10* with *lane 11*). These results assert that the APCwt-(1250–1269) peptide blocks only LP-BER without affecting SP-BER and that this blockade is linked to the pol-β-mediated strand-displacement synthesis of abasic DNA.

Colon Cancer Cells with APC Gene Knock-out Are Protected from Methylation-induced Cytotoxicity—Once we established the physical and functional interaction of APC and pol-β in the LP-BER pathway, we tested its *in vivo* implication in response to DNA-damaging agents. It is hypothesized that cells with a wild-type *APC* gene will be more sensitive to DNA-damaging agents that utilize the LP-BER pathway for the repair of damage. For these experiments, we treated HCT-116 and LoVo cell lines (which carry wild-type or mutant *APC* genes lacking the PIP-like box, respectively) with DNA-damaging agents MMS and UV light. Treatment with MMS causes methylation of DNA that is repaired by the LP-BER pathway (24), whereas UV irradiation causes DNA damage that is repaired by the nucleotide excision repair pathway (reviewed in Ref. 25). If APC blocks DNA damage-induced LP-BER and not the NER pathway, we should expect a differential sensitivity of MMS treatment *versus* UV irradiation to the HCT-116 cells. Indeed, results showed a similar possibility (Fig. 8*A*), since the growth of HCT-116 cells was retarded after treatment with MMS (Fig. 8*A*), whereas LoVo cells continue to grow in a time-dependent manner (Fig. 8*C*). These results suggest that HCT-116 cells were more sensitive to MMS treatment than the LoVo cells. On the other hand, both HCT-116 and LoVo cells were more sensitive to UV irradiation as compared with the MMS treatment (Fig. 8, *A* and *C*). The increased sensitivity in HCT-116 cells after MMS treatment is correlated with increased APC protein levels (Fig. 8*B*) that may have blocked MMS-induced LP-BER pathway (Fig. 8*D*, see increased comet signal in HCT-116 cells treated with MMS as compared with control). Since LoVo cells carry mutant APC protein levels that lack the PIP-like box, MMS-induced DNA lesions were repaired more efficiently in LoVo cells than HCT-116 cells, resulting in a better tolerance of MMS treatment in LoVo cells than HCT-116 cells (Fig. 8*D*; comet signals are the same in control and MMS-treated LoVo cells). This observation was directly tested in HCT-116 cells by RNA interference experiments. In this experiment, we knocked out *APC* gene expression by transfecting cells with pSiRNA-APC plasmid. Results showed a more than 80% reduction in the APC protein level of HCT-116 cells by this system (Fig. 9*A*). We then treated these cells with MMS for 1 h and determined

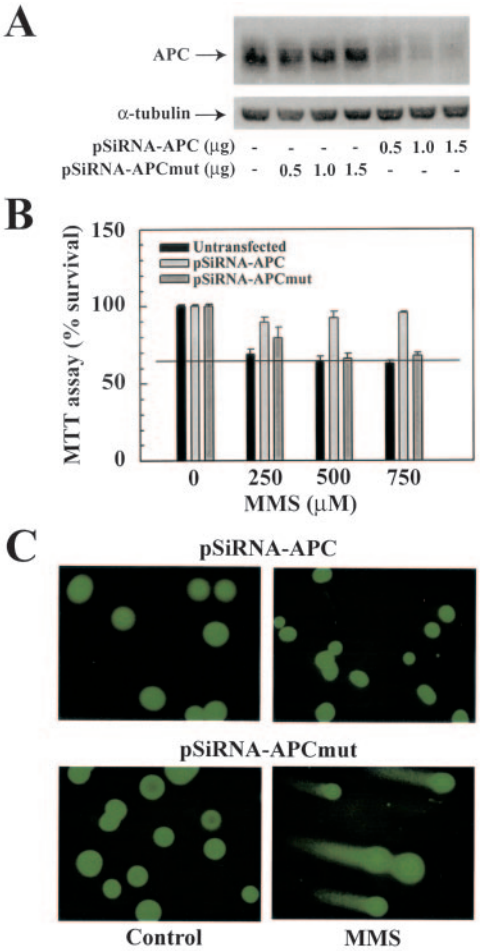

FIG. 9. **HCT-116 cells with *APC* gene knock-out are less sensitive to MMS-induced cytotoxicity.** The *APC* gene expression was knocked out in HCT-116 cells by the RNA interference technique. After transfection with pSiRNA plasmids, cells were grown in McCoy's 5A medium containing 0.5% (v/v) fetal bovine serum starved for 16 h and then treated with 500 μM MMS for 1 h. After treatment, the medium was replaced with a fresh medium containing 10% (v/v) fetal bovine serum. At different time intervals, cell survival was determined by the MTT assay. *A*, the APC protein levels in HCT-116 cells transfected with pSiRNA-APC or pSiRNA-APCmut plasmids. α-Tubulin levels served as loading control of the proteins. *B*, the reversal of growth arrest of HCT-116 cells transfected with pSiRNA-APC in response to MMS treatment. Data represent mean ± S.E. of three different experiments. *C*, comet assay. HCT-116 cells were transfected with either pSiRNA-APC or pSiRNA-APCmut plasmids and then treated with 500 μM MMS for 1 h. After 24 h of incubation, the cells were processed for comet assay as described under "Materials and Methods."

their growth after a 24-h recovery. The pSiRNA-APC-transfected cells showed resistance to MMS treatment as compared with pSiRNA-APCmut transfected or control (untransfected) cells (Fig. 9*B*), which correlated with its DNA repair capacity in the presence or absence of *APC* gene expression (Fig. 9*C*). The comet signal is not seen in pSiRNA-APC-transfected HCT-116 cells treated with MMS, which is clearly visible in pSiRNA-APCmut-transfected cells (Fig. 9*C*). From these results, we conclude that MMS-induced sensitivity to HCT-116 cells is due to an increased level of APC that blocks DNA repair by the LP-BER pathway.

Since both HCT-116 and LoVo cell lines are defective in the mismatch repair (MMR) system and they are not isogenic in origin, they may have differences in their mutational spectrum other than the *APC* gene that may also influence the MMS-induced sensitivity of these cells. To test this possibility, we used isogenic HCT-116 cell line, which has been made proficient to MMR by introducing a single copy of human chromosome 3 (HCT-116+Ch3) (26). Chromosome 3 harbors the *hMLH1* gene, which corrects MMR deficiency and microsatellite instability and is mutated in the parental HCT-116 cell line. As a control, we used HCT-116 cells in which a single copy of human chromosome 2 has been introduced (HCT-116+Ch2) (26). A similar experiment with these cell lines was performed as discussed above. The HCT-116+Ch3 and HCT-116+Ch2 cells were transfected with pSiRNA-APC or pSiRNA-APCmut and then treated with MMS for 1 h. The growth of the cells was determined after a 24-h recovery. Both HCT-116+Ch3 and HCT-116+Ch2 cell lines transfected with pSiRNA-APC showed resistance to MMS treatment as compared with pSiRNA-APCmut-transfected cells (Fig. 10). These results were similar to the parental HCT-116 cells (Fig. 9), suggesting that the MMR system is not playing any role in the APC-mediated blockage of LP-BER in these cell lines.

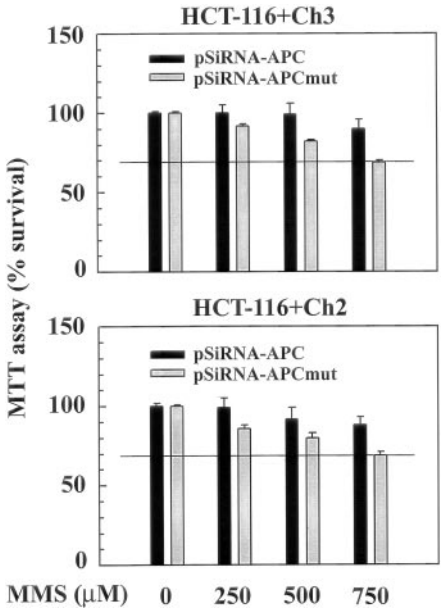

FIG. 10. **MMS-induced cytotoxicity to MMR-proficient HCT-116 cells with *APC* gene knock-out.** The *APC* gene expression was knocked out in isogenic HCT-116+Ch3 (MMR-proficient) and HCT-116+Ch2 (MMR-deficient) cell lines by the RNA interference technique. The treatment protocol was the same as described in the legend to Fig. 9. The cell survival was determined by an MTT assay. Data represent mean ± S.E. of three different estimations.

DISCUSSION

In the present investigation, we find that the APC peptide that contains a PIP-like box blocks pol-β-mediated strand displacement synthesis of LP-BER without affecting SP-BER. The effect of APC on LP-BER seems to be specific, in that pol-β-mediated 1-nucleotide incorporation and APE activity were unaffected. Most importantly, PCNA, Fen-1, and DNA ligase were unable to relieve the block. These results provide evidence that APC distinguishes between the LP-BER and SP-BER pathways and suggest a mechanism by which the LP-BER and SP-BER pathways may be regulated differentially within the cells. As the block appears to depend on the concentration of APC and pol-β, the concentration of APC and available pol-β may play a role in determining the preferential utilization of these two pathways.

The association of APC-mediated blocking of the repair of damaged DNA with susceptibility to carcinogenesis is further explored in the present study. The results provide evidence that the induction of APC by exposure to a DNA-damaging

agent, MMS, compromises the LP-BER capacity and increases sensitivity to growth of the cells. Once the APC expression is knocked out in these cells, they become less sensitive to growth after MMS treatment due to increased DNA repair capacity. It should be noted that mutant forms of APC proteins in which the PIP-like box is unaffected could contribute to the concentration-dependent APC effect, since the PIP-like box is located toward the N-terminal region of the APC protein at some distance from the mutation cluster region (amino acids 1286–1514) and the β-catenin binding site (amino acids 1342–2075). Intriguingly, these findings suggest a potential basis for the association of mutations in the *APC* gene, which is one of the earliest events in colorectal cancer, with enhanced accumulation of other mutations during the carcinogenesis cascade (reviewed in Ref. 27). Mutations of the mutation cluster region of the *APC* gene commonly produce truncated proteins that compromise the functions of APC and contribute to chromosomal instability (28, 29), yet these truncation mutations would not affect the ability of the APC to block the LP-BER pathway. Exposure to agents that are capable of enhancing the levels of APC might block the ability of these cells to repair DNA damage through the LP-BER pathway and increase the susceptibility of the colonic epithelial cell to the "second hit." Finally, in a manner analogous to the proposed use of BER inhibitors, enhancement of the concentrations of APC may enhance the efficacy of cytotoxic drugs that induce cell death by producing DNA damage (reviewed in Ref. 30).

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