The APC Tumor Suppressor Promotes Transcription-Independent Apoptosis *In vitro*

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Abstract

The APC tumor suppressor is found in nonproliferating epithelial cells of the colonic crypts and is mutated in most colorectal tumors. To understand the function of APC in normal epithelium and how its loss leads to tumor formation, we tested whether APC is a mediator of apoptosis using an *in vitro* assay that monitors caspase-3-mediated cleavage of lamin B protein or a colorimetric substrate in a cell-free *Xenopus* egg extract. Recombinant APC protein accelerates apoptosis-associated caspase activity independently of ongoing transcription and protein synthesis. Conversely, the addition of mutant APC and immunodepletion of *Xenopus* APC decelerates apoptosis-associated caspase activity. Acceleration of apoptosis by APC is abolished by the caspase-8 inhibitor Z-IETD-FMK, demonstrating that caspase-8 is an essential component of APC-mediated apoptosis. These results suggest that the induction of apoptosis may be one role of APC in tumor suppression and that this mechanism is independent of β-catenin-mediated effects on transcription. (Mol Cancer Res 2005; 3(2):78–89)

Introduction

The APC tumor suppressor is normally expressed in nonproliferating colorectal epithelium and is believed to be essential for maintaining normal growth control and differentiation (1). The colorectal epithelium is a dynamic cell population composed of several different cell types. Mitotically active stem cells reside at the base of the colonic crypts; postmitotic daughter cells differentiate and migrate along the crypt axis toward the lumen. A limited amount of programmed cell death (or apoptosis) occurs along the crypt axis, although the main population of apoptotic cells in the epithelium is observed toward the lumenal aspect of the crypts (2-4). The expression pattern of APC suggests a role for the tumor suppressor in the induction of cell death, inhibition of mitosis or cell cycle control, and/or induction of cellular differentiation. Its mutation is associated with >80% of colorectal tumors (5, 6).

The APC protein is a 310-kDa cytoplasmic protein that homodimerizes (7, 8), binds and down-regulates β-catenin (9, 10), and binds axin (11), microtubules (12, 13), EB1 (14), the human homologue of *Drosophila* discs large protein (15), and a Rac-specific guanine nucleotide exchange factor known as Asef (16). APC is phosphorylated by the kinases CDK1/p34^{cdc2} (17) and GSK3β (18). Phosphorylated APC down-regulates cytoplasmic and nuclear β-catenin levels to modulate the transcriptional profile of the cell (19, 20). The expression levels of specific genes are regulated by β-catenin and include c-*myc*, *cyclin D1*, and *Tcf1* (21-23). Other gene targets are predicted to include those that regulate growth, differentiation, and/or cell death.

Several studies in the literature suggest a link between APC and apoptosis. Induction of an *APC* transgene in a colon carcinoma cell line lacking endogenous APC increases apoptosis (24). Conversely, genetic studies in the fly show that loss of *APC* increases apoptosis in the neurons of the developing retina (25). These apparently conflicting results may reflect distinct roles of APC during development and differentiation versus tumor suppression or cell-specific responses to the expression of APC.

In this study, a cell-free apoptosis assay composed of *Xenopus laevis* egg extract and either normal mouse liver nuclei or a commercially available colorimetric substrate (26) was used to examine the role of APC in apoptosis. We show that baculovirus-expressed APC protein accelerates the apoptotic event of caspase-mediated substrate cleavage independently of ongoing transcription and protein synthesis. Additionally, exogenously added β-catenin does not change the apoptotic time course, suggesting that the ability of APC to accelerate apoptosis is independent of β-catenin. The addition of mutant APC or immunodepletion of *Xenopus* APC decelerates apoptosis-associated caspase activity. The effects of APC on apoptosis were abrogated by the caspase-8 inhibitor Z-IETD-FMK, demonstrating that caspase-8 is an essential component of APC-dependent apoptosis. These results suggest that the promotion of apoptosis may be one role of APC in tumor suppression and that this function of APC is at least in part independent of its effects on target gene expression.

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Results

Addition of APC Protein Accelerates Apoptosis in an In vitro *Assay*

To test the ability of APC to function as a direct mediator of apoptosis, cell-free *in vitro* apoptosis assays using *X. laevis* egg extracts were done (26). Soluble cytosolic and membrane fractions containing intact mitochondria are recombined with isolated normal mouse liver nuclei to measure an intrinsic program of apoptosis, which occurs as a consequence of a caspase-mediated protein cleavage cascade. Our assays use cleavage of either the 70-kDa nuclear protein lamin B to 45- and 25-kDa peptides or a commercially available colorimetric substrate by effector caspases as an indicator of apoptosis over a time course of several hours. This assay has been used successfully by others to define the roles of several apoptotic mediators, such as Bcl-2, cytochrome *c*, and caspase-8 (26-29).

Full-length APC protein was generated by baculovirus infection of Sf9 insect cells. Western blot analysis of resultant Sf9 cell lysate with a COOH- or NH_2-terminal APC-specific antibody revealed a full-length APC band of 310 kDa (Fig. 1A and B, respectively). Addition of baculovirus-expressed APC to the *in vitro* assay accelerates the timing of caspase-mediated lamin B cleavage in a dose-dependent manner (Fig. 1C-F and G-H). Total protein concentration in the assay was held constant, as was the total amount of exogenous protein. Control and APC-containing lysates were prepared from Sf9 cells infected with baculovirus carrying an empty expression vector or full-length *APC*, exons 1 to 15, respectively. Protein concentrations were titrated to reveal the effects of increasing doses of APC in the *in vitro* assay. APC-containing lysates at a final concentration of 0.3 μg/μL accelerate the timing of apoptosis by 30 minutes compared with control, whereas APC-containing lysates at a final concentration of 0.6 μg/μL accelerate apoptosis by 60 minutes. A further increase in APC-containing lysate concentration to 0.9 μg/μL also accelerates apoptosis by 60 minutes, perhaps indicating a plateau of APC-mediated temporal effects.

Purified APC protein also accelerates the timing of apoptosis-associated caspase activity in a dose-dependent manner (Fig. 1G-H). APC-containing and control Sf9 cell lysates were purified using nickel column purification and were titrated into the egg extracts such that the total protein concentration is constant. Apoptosis-associated caspase-3 activity in these experiments was measured by cleavage of a colorimetric substrate. The time of apoptosis is measured as the time at which the increase in absorbance was half-maximal. A representative trial is shown in Fig. 1G, whereas the results of three trials are summarized in Fig. 1H. Addition of purified APC to 0.067 μg/μL (Fig. 1G) accelerates apoptotic activity by 48 minutes; addition of purified APC to 0.133 μg/μL accelerates activity by 54 minutes; and addition of purified APC to 0.2 μg/μL accelerates activity by 96 minutes. Results of three independent trials (Fig. 1H) show that addition of purified APC to 0.067 μg/μL accelerates apoptotic activity by an average (SE) of 46 (2) minutes; addition of purified APC to 0.133 μg/μL accelerates activity by an average (SE) of 70 (13) minutes; and addition of purified APC to 0.2 μg/μL accelerates activity by an average (SE) of 92 (11) minutes. Thus, in this system, purified APC accelerates the timing of caspase-3 activation in a dose-dependent manner reaching saturating amounts between 0.133 and 0.2 μg/μL APC protein.

To test whether a clinically observed truncation of the APC protein in persons with familial APC could accelerate apoptosis, the protein produced from a 5-bp deletion in *APC* at codon 1,309 was expressed in Sf9 cells using baculovirus infection and purified by nickel column chromatography (Fig. 1B). When added to the egg extract, this mutant protein was unable to accelerate the timing of the apoptosis-associated increase in caspase-3 activity, instead demonstrating a slight delay of apoptosis (Fig. 1I). The timing of apoptosis is measured as the time at which the increase in caspase-3-mediated colorimetric substrate cleavage is half-maximal. In these trials, addition of purified mutant APC-1309 at 0.6 mg/mL decelerates apoptotic activity by ~45 minutes; in contrast, addition of purified wild-type APC at 0.6 mg/mL accelerates activity by ~45 minutes (Fig. 1I; data not shown).

Immunodepletion of Xenopus *APC Delays Apoptosis*

Because addition of exogenous APC accelerates the time course of apoptosis in a dose-dependent manner, we tested whether reduction of endogenous APC would conversely affect the apoptotic time course. *Xenopus* APC is detected in the cytosolic fraction but not in the membrane fraction of the egg extract by Western analysis (Fig. 2A). Therefore, APC was immunodepleted from the cytosolic fractions of extracts using anti-human APC antibodies, APC-Ab1 and APC-Ab5 (Calbiochem, La Jolla, CA). Control samples were treated with normal mouse IgG. Although APC immunodepletion was incomplete (data not shown), the partial immunodepletion of endogenous *Xenopus* APC delays the timing of caspase-mediated lamin B cleavage by 30 minutes (Fig. 2B). Caspase activity is observed at 210 minutes in the APC-depleted egg extract, 180 minutes in the IgG-depleted control, and 120 minutes in the untreated control.

APC-Mediated Acceleration of Apoptosis Is Independent of Transcription and Protein Synthesis

Although similarly prepared *Xenopus* egg extracts from laid eggs are quiescent for RNA polymerase II–dependent transcription (30), we ensured the absence of transcriptional activity by addition of the RNA polymerase II inhibitor α-amanitin (Sigma, St. Louis, MO) to a final concentration of 2 ng/μL. Control and APC-containing lysates were added to a final protein concentration of 0.9 μg/μL. The addition of APC-containing lysates accelerates the timing of apoptosis-associated caspase-mediated lamin B cleavage from 240 to 180 minutes; this activity is not affected by α-amanitin (Fig. 3A-D). These experiments show that the ability of APC to accelerate apoptosis in this system is transcription independent. *Xenopus* egg extracts are prepared in the presence of cyclohexamide; therefore, APC-mediated effects on apoptosis occur in the absence of protein synthesis.

Addition of β-Catenin Does Not Affect the Timing of Apoptosis

APC is well known for its ability to facilitate degradation of the signal transduction molecule β-catenin (19, 20). Therefore, we asked whether manipulation of β-catenin protein levels in the extracts would alter the timing of apoptosis. Full-length

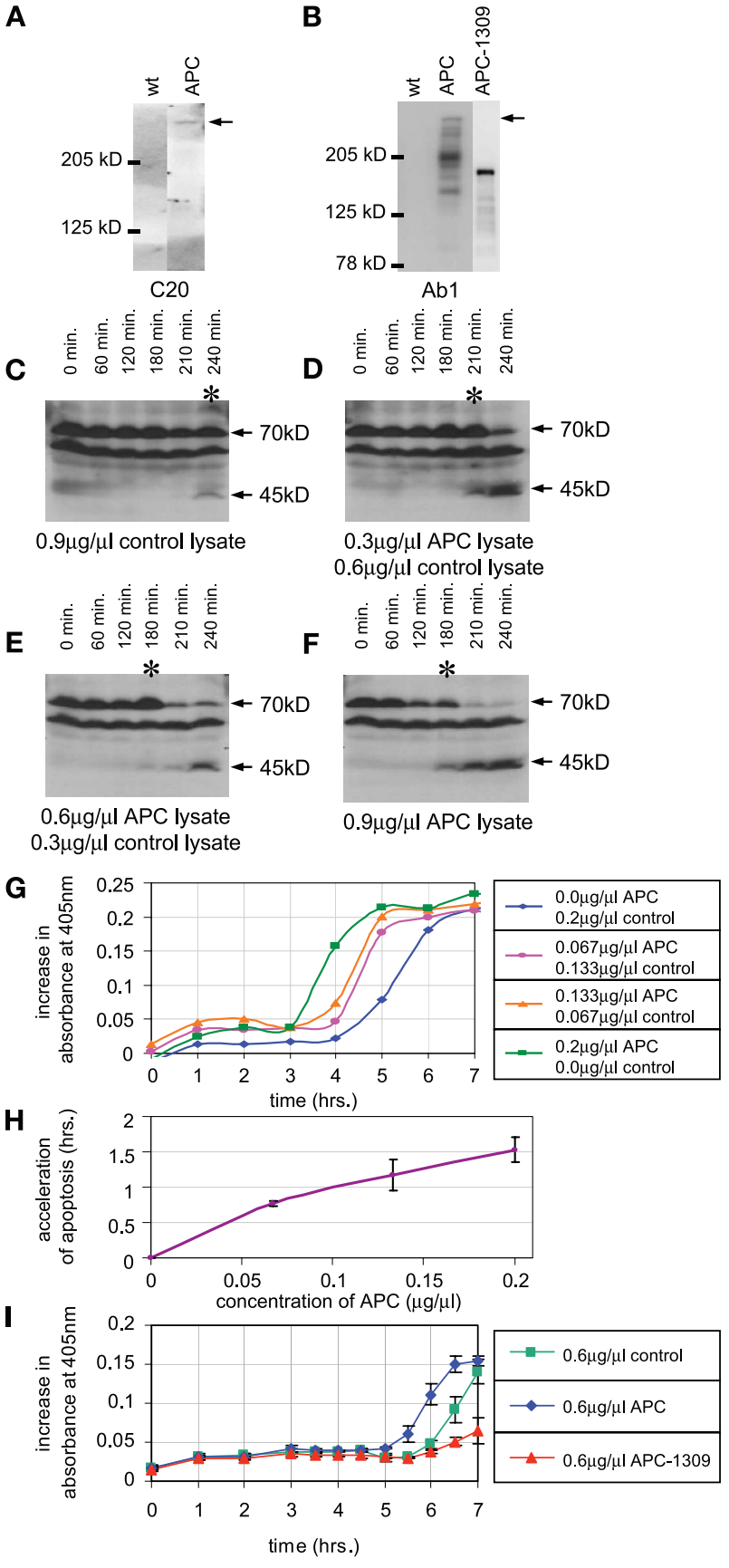

FIGURE 1. APC accelerates apoptosis in a dose-dependent manner *in vitro*. **A** and **B.** Western blots of insect cell lysates probed with an anti-human-APC COOH-terminal (C-20) or NH_2-terminal (Ab1) antibody, respectively, show expression of full-length APC or a mutant APC-1309 using a baculovirus expression system. Lanes contain lysate from Sf9 cells infected by wild-type baculovirus (*wt*), baculovirus carrying *APC*, or baculovirus carrying the mutant *APC-1309*. *Arrow,* full-length APC at 310 kDa. **C-F.** Western blots probed with anti–lamin B antibodies show effector caspase activity at specific time points *in vitro* by cleavage of full-length lamin B (70 kDa) to 25- and 45-kDa fragments. The 70-kDa full-length lamin B and the 45-kDa fragment are recognized by the antibody used. The assay contains 1% *Xenopus* egg extract membrane fraction. Sf9 cells were infected by baculovirus carrying either an empty vector or *APC*, generating control and APC-containing lysates, respectively. *Asterisks,* lanes where lamin B cleavage first occurs detectably. **C.** Control lysate, 0.9 µg/µL. **D.** APC-containing lysate, 0.3 µg/µL; control lysate, 0.6 µg/µL. **E.** APC-containing lysate, 0.6 µg/µL; control lysate, 0.3 µg/µL. **F.** APC-containing lysate, 0.9 µg/µL. **G.** Representative results from *in vitro* assay displayed as an increase in absorbance at 405 nm versus time in hours in which apoptosis is measured by cleavage of a colorimetric substrate by activated caspase-3. The assay contains 1% *Xenopus* egg extract membrane fraction. Control and APC-containing Sf9 cell lysates were purified and added at the concentrations indicated to achieve a final combined concentration of 0.2 µg/µL. **H.** Acceleration of apoptosis in hours as measured by cleavage of a colorimetric substrate by activated caspase-3 versus concentration of purified APC (µg/µL). Timing of apoptosis is measured as the time in hours at half-maximal increase in absorbance at 405 nm. Result of three independent experiments, one of which is shown in **G**; bars, SE. **I.** Acceleration of apoptosis assayed by colorimetric substrate cleavage as in **G**. Control, APC, and mutant APC-1309 purified cell lysates were added to the final concentrations indicated. Representative of three independent experiments. Result of triplicate samples within each experiment; bars, SE.

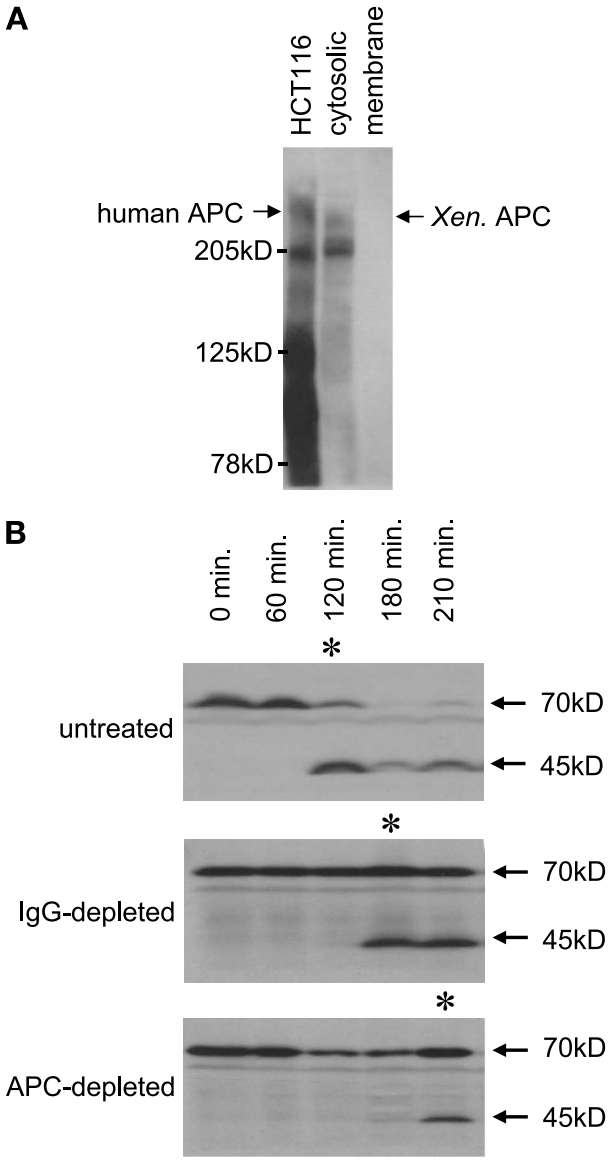

FIGURE 2. Immunodepletion of *Xenopus* APC delays apoptosis *in vitro*. **A.** Western blot probed with an antibody to the NH_2 terminus of human APC, Ab1, shows *Xenopus* APC in the cytosolic fraction of egg extracts but not in the membrane fraction. Lanes contain lysates from the human colon carcinoma cell line HCT116 with full-length APC as control, 100 μg protein cytosolic fraction of *Xenopus* egg extract, and 20 μg protein membrane fraction of *Xenopus* egg apoptotic extract. *Arrow,* full-length 310-kDa human APC and *Xenopus* APC (*Xen. APC*). **B.** Western blots probed with anti–lamin B antibody show effector caspase activity at specific time points *in vitro*. *Xenopus* APC was partially depleted from the cytosolic fraction of the egg extract using a mixture of two monoclonal NH_2-terminal-specific APC antibodies and Pansorbin. IgG-depleted control was matched for antibody concentration and volume, whereas the untreated sample was incubated on ice without the addition of antibodies or a correction of volume. The assay contains 1% *Xenopus* egg extract membrane fraction. *Asterisks,* lanes where lamin B cleavage first occurs.

human β-catenin was generated using baculovirus infection of Sf9 insect cells. Western analysis of resultant Sf9 cell lysate with a COOH-terminal β-catenin-specific antibody reveals a full-length β-catenin band of 95 kDa (Fig. 4A). Addition of Sf9 cell lysates containing baculovirus-expressed β-catenin does not change the timing of apoptosis (Fig. 4B). Exogenous β-catenin is present at high levels throughout the experiment, as shown by Western blots in Fig. 4B stripped of lamin B antibodies and reprobed with antibodies specific for human β-catenin (Fig. 4C). At different exposure times, a 95-kDa *Xenopus* β-catenin band is present in all lanes due to cross-reactivity of the anti-human β-catenin antibody.

Z-IETD-FMK Prevents APC-Induced Acceleration of Apoptosis

To place APC in a known apoptotic pathway, inhibitors of two major initiator caspases, caspase-8 and caspase-9, were used to examine their effects on APC-mediated apoptosis. These competitive inhibitors, Z-IETD-FMK and Z-LEHD-FMK, comprise consensus tetrapeptide recognition sites specific for their corresponding caspase over a range of concentrations (31, 32).

Western analysis using an anti-caspase-8-specific antibody identifies *Xenopus* caspase-8 in the cytosolic fraction of egg extracts (Fig. 5A). This polyclonal antibody recognizes human pro-caspase-8 at 55 kDa and the activated form of caspase-8 at 20 kDa. Both forms of *Xenopus* caspase-8 are present in the cytosolic fraction of the egg extracts, whereas the membrane fraction shows a faint band at 55 kDa. Western analysis of lysates from normal mouse liver nuclei reveals a light band corresponding to the 55-kDa unactivated form of caspase-8. As a control, Western analysis of the human colon carcinoma cell line HCT116 lysate reveals a caspase-8-specific band at 55 kDa.

Inhibition of caspase-8 dramatically decreases the effect of unpurified APC on apoptosis in a dose-dependent manner

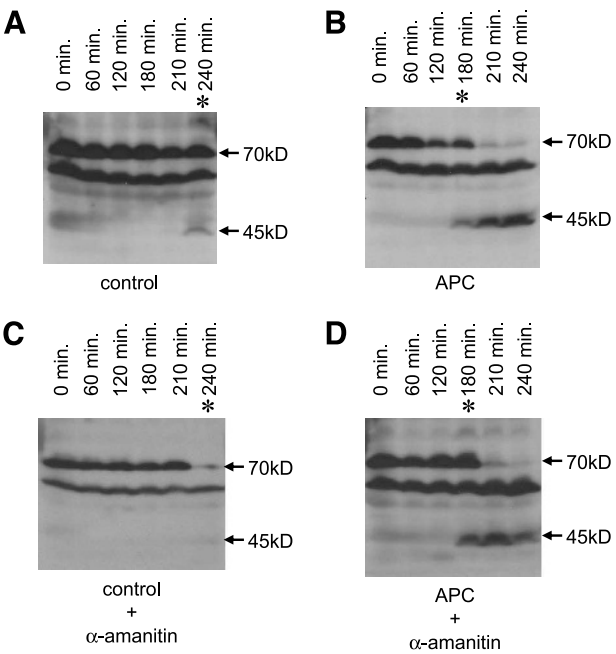

FIGURE 3. APC-mediated acceleration of apoptosis *in vitro* is transcription independent. **A-D.** Western blots probed with anti–lamin B antibody show effector caspase activity at specific time points *in vitro*. *Asterisks,* lanes where lamin B cleavage first occurs. **C** and **D.** The RNA polymerase II inhibitor α-amanitin was added to a final concentration of 2 ng/μL.

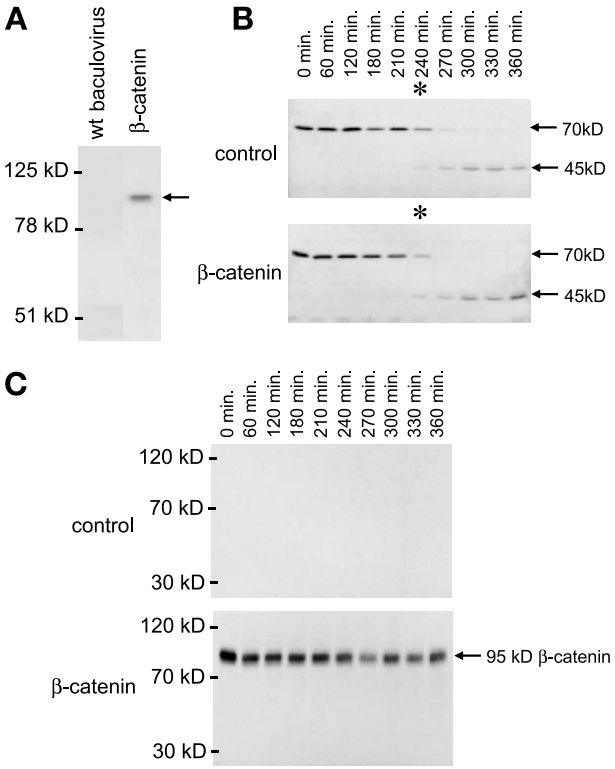

FIGURE 4. β-Catenin does not alter the timing of apoptosis *in vitro*. **A.** Western blot probed with an anti-human β-catenin COOH-terminal antibody shows expression of full-length β-catenin in the baculovirus expression system. Lanes contain lysates from Sf9 cells infected by wild-type baculovirus or baculovirus carrying *β-catenin*. *Arrow,* full-length β-catenin at 95 kDa. **B.** Western blots probed with anti–lamin B antibody show caspase activity at specific time points *in vitro* following addition of β-catenin. The assay contains 0.6% *Xenopus* egg extract membrane fraction. Sf9 cells infected by wild-type baculovirus or baculovirus carrying *β-catenin* generated control or β-catenin-containing protein lysates that were added to a final protein concentration of 0.85 μg/μL. *Asterisks,* lanes where lamin B cleavage first occurs. **C.** Western blots from **B** were stripped and probed with anti-β-catenin antibody to show high levels of β-catenin throughout the time course of the experiment.

(Fig. 5B-D). The caspase-8 inhibitor used here, Z-IETD-FMK, is specific for caspase-8 and has an IC_{50} for caspase-8 in cellular extracts of 1 nmol/L (31). Addition of Z-IETD-FMK to a final concentration of 2 nmol/L decreases APC-mediated acceleration of lamin B cleavage by 50%. Increasing the inhibitor to a final concentration of 10 nmol/L completely abolishes the acceleration of apoptosis by APC (Fig. 5D).

Western analysis using an anti-caspase-9-specific antibody identifies *Xenopus* caspase-9 in the cytosolic fraction of egg extracts (Fig. 6A). This polyclonal antibody recognizes human pro-caspase-9 at 50 kDa and the activated form of caspase-9 at 10 kDa. Analysis of egg extracts shows a protein in the cytosolic fraction at ~10 kDa and another cross-reactive protein slightly larger than 10 kDa. Western analysis of lysates from normal mouse liver nuclei and the membrane fraction of *Xenopus* egg extracts do not reveal any cross-reactive proteins. As a control, Western analysis of the human colon carcinoma cell line HCT116 total cell lysate identifies a caspase-9-specific band at ~50 kDa and a band at ~35 kDa.

In contrast to our results with the caspase-8 inhibitor, inhibition of caspase-9 does not abolish the acceleration of apoptosis by unpurified APC (Fig. 6B-D). Addition of the caspase-9 inhibitor Z-LEHD-FMK to a final concentration of 2 nmol/L does not alter the effects of APC on caspase-mediated

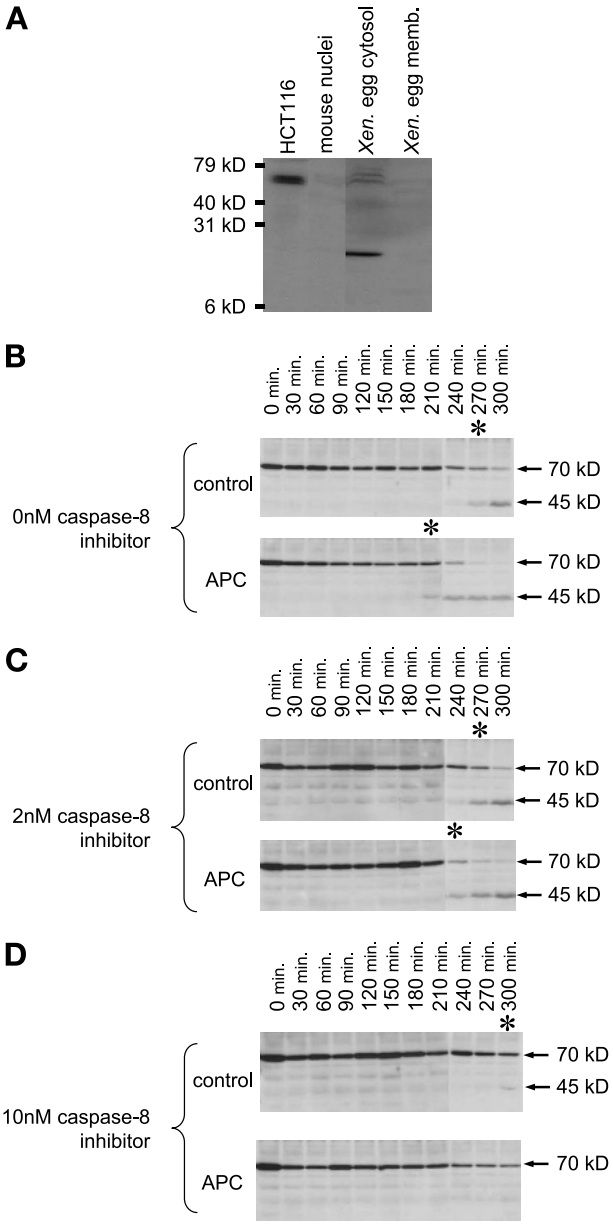

FIGURE 5. Inhibition of caspase-8 abolishes the acceleration of apoptosis by APC. **A.** Western blot probed with anti-caspase-8 identifies caspase-8 proteins in *Xenopus* egg extract cytosolic fraction. **B-D.** Western blots probed with anti–lamin B antibody show effector caspase activity at specific time points *in vitro* following the addition of a caspase-8 inhibitor. The assay contains 1% *Xenopus* egg extract membrane fraction. Cell lysates were added to a final protein concentration of 0.85 μg/μL. *Asterisks,* lanes where lamin B cleavage first occurs. **B.** No caspase inhibitor was added. **C.** The caspase-8 inhibitor Z-IETD-FMK was added to a final concentration of 2 nmol/L. **D.** The caspase-8 inhibitor Z-IETD-FMK was added to a final concentration of 10 nmol/L. The 45-kDa lamin B cleavage product at the 300-minute time point in extracts containing APC and 10 nmol/L Z-IETD-FMK is 1.5-fold higher than background but does not meet our criteria for apoptosis.

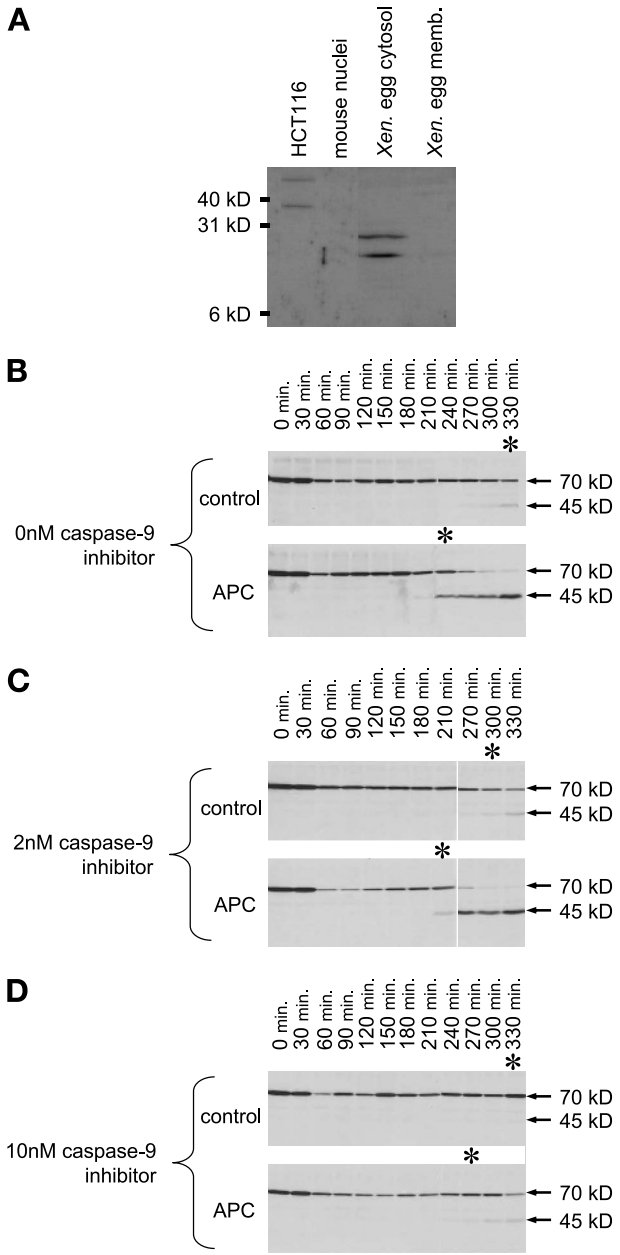

FIGURE 6. APC-mediated acceleration of apoptosis is not abolished by inhibition of caspase-9. **A.** Western blot probed with anti-caspase-9 shows the presence of anti-caspase-9 reactive proteins in *Xenopus* egg extract cytosolic fraction. **B-D.** Western blots probed with anti–lamin B antibody show effector caspase activity at specific time points *in vitro* following addition of a caspase-9 inhibitor. The assay contains 1% *Xenopus* egg extract membrane fraction. Sf9 cells infected by wild-type baculovirus or baculovirus carrying *APC* generated control or APC-containing protein lysates that were added to a final concentration of 0.85 μg/μL. *Asterisks,* lanes where lamin B cleavage first occurs. **B.** No caspase inhibitor was added. **C.** The caspase-9 inhibitor Z-LEHD-FMK was added to a final concentration of 2 nmol/L. **D.** The caspase-9 inhibitor Z-LEHD-FMK was added to a final concentration of 10 nmol/L.

lamin B cleavage. Even at a final concentration of 10 nmol/L Z-LEHD-FMK, APC accelerates apoptosis relative to controls, although both control and experimental assays are delayed slightly at this high concentration of inhibitor.

APC Accelerates Apoptosis with Low Levels of Exogenous Cytochrome c and in the Absence of Mitochondria

Cytochrome *c* levels were manipulated *in vitro* to ask whether the effect of APC on the apoptotic time course is mediated independently of cytochrome *c* release from the mitochondria. An experimental time course without mitochondria or cytochrome *c* did not show any apoptotic activity on addition of either control or APC-containing lysates (Fig. 7A). Lamin B was monitored for cleavage for up to 11 hours. In contrast, addition of exogenous cytochrome *c* at a low level (0.02 μmol/L) produces apoptosis-associated lamin B cleavage in both control and APC samples. Lysates containing APC accelerate lamin B cleavage by 2 hours compared with control lysates (Fig. 7B). Addition of higher levels of cytochrome *c* (0.2 μmol/L) results in apoptosis-associated lamin B cleavage in both control and APC time courses at the 1-hour time point (Fig. 7C).

Discussion

The biology of the colorectal epithelium dictates that cell turnover occurs to maintain homeostasis and proper tissue function. The loss of full-length APC in neoplastic colorectal epithelial cells could decrease apoptotic cell death, increase cell number, and promote the development and progression of tumors. This connection is underscored by the observation that adenomas from APC patients show greatly increased numbers of both apoptotic bodies and cycling cells compared with normal crypts (33). Additionally, the relationship between cell cycle and cell death in the context of carcinogenesis becomes more intimate with the demonstration that APC blocks cell cycle progression (34, 35). It is clear that APC can affect several distinct variables determining tissue homeostasis and, by extension, to suppress tumor formation by regulating choices between cell growth and cell death.

Our experiments show that human APC protein accelerates apoptosis *in vitro* as measured by a time course of caspase-mediated lamin B cleavage. APC-mediated effects on the rate of apoptosis were direct and titratable over a range of concentrations. Additionally, partial immunodepletion of endogenous *Xenopus* APC from the egg extract or the addition of a truncated version of human APC delays the intrinsic, programmed time course of apoptosis. The truncated APC protein was designed to resemble common germ line and somatic mutations in the *APC* gene associated with familial APC and sporadic colorectal cancers, respectively. It is unclear whether the delay in the apoptotic time course associated with truncated APC reflects the ability of the mutant to interact with full-length *Xenopus* APC through a dominant-negative interaction or another independent mechanism. It is interesting to speculate, however, that there may be subtle effects of heterozygosity for APC in the colonic crypts of persons with familial APC or in mouse models of familial APC.

Current models of APC function state that APC promotes β-catenin degradation in the cell and therefore opposes the signal transduction and transcriptional cofactor roles of β-catenin (19, 20). We examined whether transcription regulation plays a role in the acceleration of apoptosis by APC *in vitro*. Our experiments show that the acceleration of

apoptotic events by APC is independent of its role in β-catenin-mediated transcription. A previous study showed that induction of an *APC* transgene in the HT-29 colon carcinoma cell line lacking full-length APC increased apoptosis from 0.3% to 3% of the cells (24). Cell culture systems, however, do not differentiate between transcription-dependent and transcription-independent effects on apoptosis. Similarly, there may be transcription-dependent effects of APC on apoptosis that we were unable to show using these *in vitro* techniques.

In addition to a role as a transcriptional cofactor, β-catenin performs signal transduction functions in Wnt-regulated pathways. Wnt signaling is regulated in part by β-catenin protein degradation through ubiquitination and proteasome-mediated degradation, an activity up-regulated by APC (19, 20). *Xenopus* egg extracts have been used to reconstitute proteasome activity and interactions of the cytoplasmic components of Wnt pathways (36, 37). Therefore, we tested whether β-catenin could affect apoptosis directly. Addition of exogenous β-catenin had no effect on the timing of apoptosis (Fig. 4B), although high levels of β-catenin were present at all time points (Fig. 4C). Thus, APC may have the ability to up-regulate apoptosis independent of β-catenin protein levels.

The effects of post-translational modifications of APC and/or β-catenin in our assay remain uncharacterized. Baculovirus expression of protein was used to maximize proper protein folding and post-translational modification; however, the phosphorylation status of baculovirus-expressed APC and β-catenin is unknown. This may be biologically relevant because APC contains numerous consensus and near-consensus phosphorylation sites for several kinases and is phosphorylated *in vivo* by CDK1/p34^{cdc2} (17) and GSK3β (18).

Baculovirus-expressed, full-length human APC was evaluated by Western analysis with antibodies specific for the NH_2 and COOH termini of APC. Full-length protein is observed at 310 kDa with both antibodies. Additional bands are observed at ~80 to 200 kDa, representing NH_2-terminal-specific truncated products. Therefore, it is unknown whether full-length or smaller forms of APC are responsible for the proapoptotic activity observed. A 90-kDa cross-reactive APC fragment has been observed in epithelial cell lines undergoing apoptosis (38). This 90-kDa fragment is formed by cleavage at Asp^{777} by a group II caspase similar or identical to caspase-3. It is unclear whether a 90-kDa caspase-generated APC cleavage product further mediates an apoptotic process in these cell lines or is

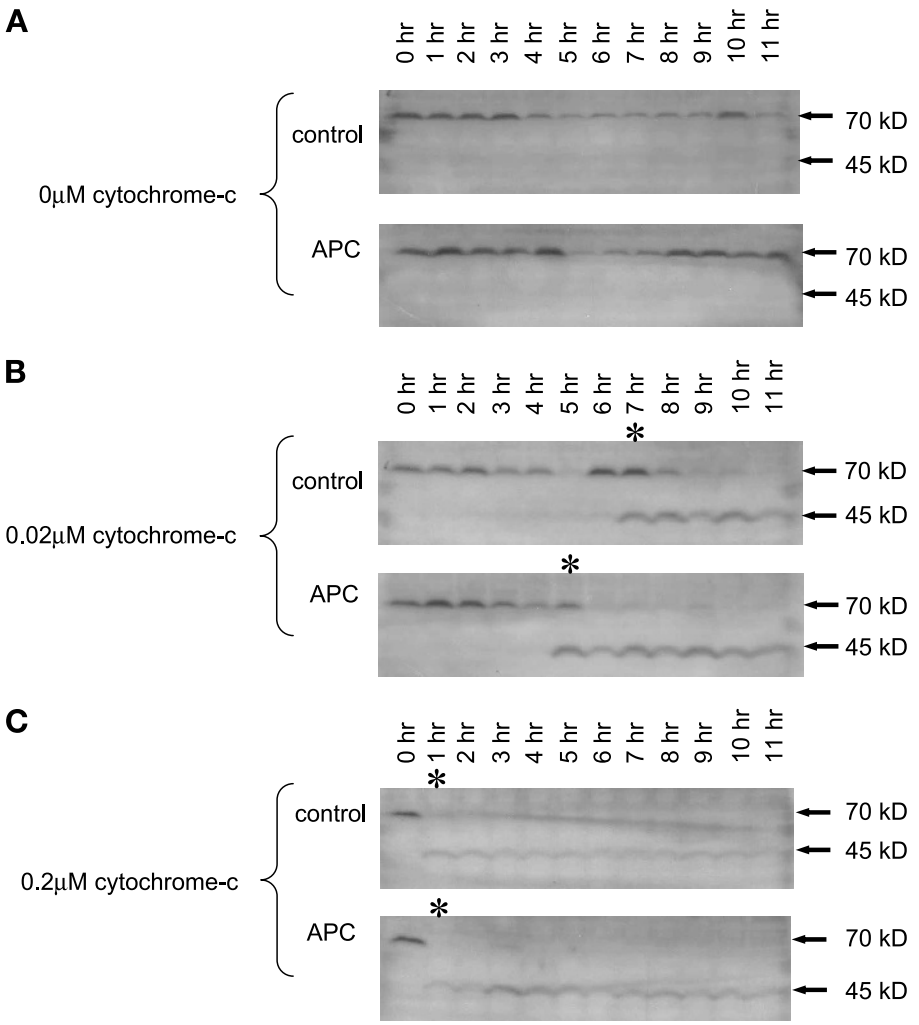

FIGURE 7. APC accelerates apoptosis in the presence of low levels of exogenous cytochrome *c* and the absence of mitochondria. Western blots probed with anti–lamin B antibody show effector caspase activity at specific time points *in vitro* following addition of cytochrome *c*. The assay does not contain *Xenopus* egg extract membrane fraction. Sf9 cells infected by wild-type baculovirus or baculovirus carrying *APC* generated control or APC-containing protein lysates that were added to a final concentration of 0.85 μg/μL. *Asterisks,* lanes where lamin B cleavage first occurs. **A.** No cytochrome *c* added. **B.** Cytochrome *c* added to a final concentration of 0.02 μmol/L. **C.** Cytochrome *c* was added to a final concentration of 0.2 μmol/L.

itself a by-product of apoptosis. Western blots from our control and APC experiments were stripped of lamin B antibodies and reprobed with antibodies specific for the NH_2 terminus of APC. A 90-kDa form of APC was observed at all time points in addition to other cross-reactive forms between 80 and 200 kDa (data not shown).

The presence of two of the major initiator caspases, caspase-9 and caspase-8, was verified in *Xenopus* extracts; each was inhibited individually to determine their roles in APC-mediated apoptosis. Different concentrations of Z-LEHD-FMK and Z-IETD-FMK were tested to ensure specificity in egg extracts. The timing of lamin B cleavage is delayed on addition of the caspase-9 inhibitor Z-LEHD-FMK at a high concentration, although the acceleration of apoptosis by APC compared with controls is not altered (Fig. 6B and C). In contrast, addition of the caspase-8 inhibitor Z-IETD-FMK at 10 nmol/L completely prevents the acceleration of apoptosis by APC, leaving control time courses unchanged. These data suggest that APC mediates apoptosis via a caspase-8 pathway. Z-IETD-FMK is highly specific for caspase-8 and has an IC_{50} in cellular extracts of 1 nmol/L (31); the concentrations used here are $2\times$ IC_{50} and $10\times$ IC_{50} in cellular extracts. It is formally possible that Z-IETD-FMK inhibits caspase-6 at 10 nmol/L, as the IC_{50} for caspase-6 in cellular extracts is 5.6 nmol/L for Z-IETD-FMK (31). Inhibition of caspase-6, one of the effector caspases that cleave lamin B, would likely change the timing of lamin B cleavage in both control and APC experiments identically (similar to the small nonspecific effects we observed at high concentrations of Z-LEHD-FMK). Inhibition of caspase-8 by Z-IETD-FMK at 2 nmol/L decreases the acceleration of apoptosis by APC by 30 minutes or $\sim$50% of the expected APC-mediated acceleration of apoptosis. It is also formally possible that Z-IETD-FMK has an uncharacterized inhibitory effect on other proteins important for apoptosis. However, we feel that such an effect would be similarly unlikely to inhibit only the APC-containing experiments.

Caspase-8 is an initiator caspase involved in receptor-mediated apoptosis pathways (39). Pro-caspase-8 is activated by its recruitment to the cell surface through interaction with the death effector domain of the protein Fas-associated death domain. Pro-caspase-8 is then converted into its active form by self-processing, which in turn leads to the activation of downstream effector caspases. A classic example of a caspase-8 activation pathway is the association of the Fas receptor and Fas ligand, with the resultant induction of apoptosis. Although the main function of Fas receptor and Fas ligand is to mediate positive and negative selection of T lymphocytes, Fas-mediated cell death occurs in epithelial cells of the skin in response to sunburn, presumably as prophylaxis against acquisition of mutation by UV radiation (40). This implies that receptor-mediated pathways of apoptosis can be involved in the prevention of carcinogenic processes in epithelial cells.

Smaller forms of both caspase-8 and caspase-9 in *Xenopus* egg extracts suggest that these caspases, or a subset of these caspases, may be present in activated form. The presence of these small forms of caspase-8 and caspase-9 may also explain the apoptotic activity intrinsic to these extracts. The ability of APC to accelerate a caspase-8-dependent apoptotic cascade could then occur via modulation of a complex that includes activated caspase-8 and an inhibitor of caspase-8 that acts downstream of pro-caspase-8 cleavage. Additionally, it is unlikely that activated caspase-8 and/or caspase-9 would alter the effectiveness of the caspase inhibitors used here, as the inhibitors Z-IETD-FMK and Z-LEHD-FMK function competitively by providing the downstream target sequence in a peptide form. Although these inhibitors interact with human caspases, characterized members of the *Xenopus* caspase family have a high degree of homology with their human counterparts, with identical active sites in both human and *Xenopus* caspase-8 and caspase-9 (41).

We next investigated the effect of APC on caspase-8-dependent, mitochondria-independent apoptosis. Similar *Xenopus* egg extracts require either mitochondria or exogenous cytochrome *c* to produce an apoptotic signal (26, 28, 42). However, apoptosis-associated DNA degradation was induced in extracts in the absence of mitochondria or exogenous cytochrome *c* on addition of active caspase-8 (29), suggesting that a caspase-8-dependent, mitochondria-independent apoptosis pathway exists. The APC-mediated acceleration of apoptosis in the presence of cytochrome *c* and the absence of mitochondria, shown in Fig. 7, suggests that APC may exert its effects independently of cytochrome *c* or downstream of this step in the apoptotic cascade.

Our results contrast with those of Ahmed et al. (25), in which genetic loss of *Drosophila* APC induces neuronal apoptosis in the developing retina. This apoptosis is due to an increase in β-catenin, as the phenotype is rescued by reducing *Drosophila β-catenin* gene dosage by half. These results suggest a transcription-dependent mechanism of APC function in apoptosis. However, cell type specificity and its influence on apoptotic regulation may also account for differences between these experiments and our own.

In this study, we tested whether APC is a direct modulator of apoptosis in an *in vitro* assay using *X. laevis* egg extract competent for apoptosis. The tumor suppressor APC accelerates apoptosis-associated caspase activity and does so by a transcription-independent mechanism. The timing and efficiency of apoptosis was up-regulated by APC in a dose-dependent manner. The modulation of apoptosis was abolished by the caspase-8 inhibitor Z-IETD-FMK, demonstrating that caspase-8 is an essential component of APC-mediated apoptosis. Furthermore, APC accelerates apoptosis in the presence of low levels of exogenous cytochrome *c* and the absence of mitochondria, suggesting that APC may exert its effects independently of the release of cytochrome *c* from the mitochondria. These results show that the induction of appropriate apoptosis maybe one role of APC in tumor suppression and that this mechanism is independent of β-catenin effects on transcription.

Materials and Methods

Protein Expression and Purification

Full-length and mutant human APC proteins and full-length human β-catenin protein were expressed using a baculovirus expression system and Sf9 insect cells. Full-length *β-catenin* cDNA was a gift from Paul Polakis (Genentech, Inc., San Francisco, CA). To express untagged protein, cDNAs were

cloned directionally into the pVT-Bac transfer vector (43) using *Bam*HI and *Not*I; control vector did not contain an insert. Sf9 insect cells were cotransfected with the transfer vector and BaculoGold baculovirus (PharMingen, San Diego, CA). His-tagged APC containing a 6× histidine tag at the NH_2 terminus of the protein was expressed by cloning the cDNA into the *pFB-HTa* transfer vector (Life Technologies, Rockville, MD) using *Bam*HI and *Not*I; the control vector did not contain an insert. Mutant APC-1309 protein was created by PCR-mediated cloning of DNA encoding the first 1,309 amino acids of APC into *pFB-HTa* using *Bam*HI and *Sac*I. The cDNA insert was amplified using the upstream primer 5′-TGTAAAACGACGGCCAGTGGATCCAATGGCTGCAGCT-TCATATGATCAG-3′ and the downstream primer 5′-CTTCA-GAGCTCCTAGTTCCAATCTTTTATTTCTGC-3′. This construct recreates the open reading frame of one of the more common germ line mutant *APC* alleles in persons with familial APC that includes a 5-bp deletion at codon 1,309 and expresses a truncated protein identical to that found in these affected persons. Proteins were expressed using the Bac-to-Bac expression system following baculovirus infection of Sf9 cells (Life Technologies). Recombinant virus was amplified by passage in Sf9 cells at a multiplicity of infection of ~0.1, multiplicity of infection defined as the plaque forming unit to Sf9 cell ratio. Protein was expressed by infection of Sf9 cells at a multiplicity of infection of 3. Recombinant baculoviruses were amplified in Sf9 insect cells (PharMingen). Untagged proteins were expressed and harvested from a 75 cm^2 tissue culture flask into 0.5 mL of lysate buffer [20 mmol/L HEPES (pH 7.4), 30 mmol/L KCl, 1 mmol/L EDTA, 2% CHAPS, 1 mmol/L DTT, 0.2 mmol/L *p*-aminoethylbenzenesulfonyl fluoride HCl, 10 μg/mL leupeptin, 1 μg/mL aprotinin, 1mmol/L benzamidine], incubated 15 minutes on ice, and dialyzed against PBS for 16 hours at 4°C (26). Aliquots were stored at −80°C.

Purified APC proteins were expressed and harvested from Sf9 suspension culture using 5 mmol/L imidazole, 500 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.9), 0.1% NP40, 0.2 mmol/L *p*-aminoethylbenzenesulfonyl fluoride HCl, 10 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 mmol/L benzamidine. Lysates were sonicated on ice and centrifuged at 39,000 × *g* for 20 minutes at 4°C. Proteins were purified using a His-bind metal chelation resin (Novagen, Madison, WI) by incubation with 2.5 mL charged resin for 1 hour at 4°C with rotation. The resin was washed with 10 volumes of 60 mmol/L imidazole, 500 mmol/L NaCl, and 50 mmol/L Tris-HCl (pH 7.9) and eluted with 100 mmol/L imidazole increasing stepwise to 500 mmol/L imidazole, 500 mmol/L NaCl, and 20 mmol/L Tris-HCl (pH 7.9). Elution steps were pooled, dialyzed against 150 mmol/L NaCl and 50 mmol/L Tris-HCl (pH 7.9), and concentrated using Centriprep-10 (Millipore, Bedford, MA).

Western analysis of Sf9 cell lysates containing human APC were done by separating proteins by electrophoresis through 3% agarose horizontal gels and electroblotting onto Immobilon-P polyvinylidene difluoride membrane (Millipore). Blots were blocked by immersion in 1× TBS [20 mmol/L Tris base, 137 mmol/L NaCl (pH 7.6), 1% casein blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN)] for 1 hour at room temperature and probed with 500 ng/mL anti-APC-Ab1 or 50 ng/mL anti-APC C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) in 1× TBS (pH 7.6), 0.5% casein blocking reagent for 1 hour at room temperature. Blots were washed twice with TBST (20 mmol/L Tris base, 137 mmol/L NaCl, 0.05% Tween 20) and probed with 250 ng/mL peroxidase-labeled goat anti-mouse secondary antibody (Kirkegaard and Perry, Gaithersburg, MD) in 1× TBS (pH 7.6), 0.5% casein blocking reagent for 1 hour at room temperature. Blots were washed four times with TBST and developed using Renaissance chemiluminescence reagents (NEN, Boston, MA). Western analysis of Sf9 cell lysates containing human β-catenin was done as described above using 62 ng/mL anti-β-catenin (Transduction Laboratories, San Diego, CA).

Uninfected and baculovirus-infected insect cell cultures contain proteins that affect apoptosis (44-49). Because unpurified exogenous APC was used in some of our experiments, these baculovirus-encoded and insect cell–related proteins are present in both the control Sf9 cell lysates and those containing APC. Additionally, wild-type baculovirus carry a gene encoding a baculoviral coat protein that was deleted in the vector-only control preparation or replaced by the *APC* gene in test preparations (50). This structural protein does not change the timing of apoptosis in our experiments (data not shown). Control lysates prepared from insect cells carrying wild-type baculovirus or vector-only baculovirus were used interchangeably.

Cytochrome *c* from horse heart (Sigma) was prepared as a 100 mmol/L stock solution in water and stored at −80°C. Cytochrome *c* was diluted in water to a working solution and added to *Xenopus* egg extracts immediately before use.

Preparation of Apoptotic X. laevis *Egg Extract*

X. laevis females were injected with 100 units pregnant mare serum gonadotropin (Calbiochem). Twenty-one days later, egg laying was induced by injection of 800 units human chorionic gonadotropin (Sigma; refs. 26, 51). *Xenopus* egg extract was prepared according to Newmeyer et al. (26) and von Ahsen et al. (50) with the following modifications. Eggs were collected, resuspended in lysis buffer [250 mmol/L sucrose, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 50 μg/mL cyclohexamide, 5 μg/mL cytochalasin B, 20 mmol/L HEPES-HCl (pH 7.5)] and centrifuged at 10,000 × *g* for 12 minutes at 4°C. The cytoplasmic fraction was removed from pigment granules and yolk and then separated into cytosolic and membrane fractions by centrifugation at 200,000 × *g* for 2 hours at 4°C. The membrane fraction was removed, added to membrane wash buffer [50 mmol/L KCl, 250 mmol/L sucrose, 2.5 mmol/L $MgCl_2$, 20 mmol/L HEPES-HCl (pH 8.0), 1 mmol/L DTT, 1 mmol/L ATP, 0.2 mmol/L *p*-aminoethylbenzenesulfonyl fluoride HCl, 10 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L benzamidine], and collected as a pellet after centrifugation at 40,000 × *g* for 30 minutes at 4°C. The cytosolic and membrane fractions were stored separately in aliquots at −80°C and later combined to make an extract capable of supporting apoptotic events.

Time points at which baseline caspase-mediated lamin B cleavage occurred in different experiments were manipulable and therefore were not always identical. Ratios of membrane and cytosolic fractions for baseline control time courses of

caspase-mediated lamin B cleavage were determined empirically for each prepared extract. Membrane fractions of egg extract include intact mitochondria that influence the timing of apoptotic events. For each experiment, reactions and controls were done in parallel with a single extract preparation.

Isolation of Normal Mouse Liver Nuclei

Livers from normal adult mice were removed according to Institutional Animal Care and Use Committee–approved protocols. Tissues were prepared as described by Gorski et al. (52). The livers were rinsed in ice-cold PBS containing 0.5 mmol/L phenylmethylsulfonyl fluoride (Sigma), transferred to homogenization buffer [2 mol/L sucrose, 10 mmol/L HEPES (pH 7.6), 25 mmol/L KCl, 0.15 mmol/L spermine, 0.5 mmol/L spermidine, 1 mmol/L EDTA, 10% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L DTT], and minced with scissors. Tissues were Dounce homogenized on ice and layered onto a 2 mol/L sucrose cushion. Intact nuclei were enriched by centrifugation at 120,000 × *g* for 35 minutes at 0°C. Pellets containing intact nuclei were resuspended in nuclei storage buffer [250 mmol/L sucrose, 80 mmol/L KCl, 15 mmol/L NaCl, 15 mmol/L HEPES (pH 7.6), 5 mmol/L EDTA, 7 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.2 mmol/L *p*-aminoethylbenzenesulfonyl fluoride HCl, 10 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L benzamidine] and stored in aliquots at −80°C. Normal mouse liver nuclei are the source of lamin B.

Cell-Free Apoptosis Assays Using X. laevis *Egg Extract*

Egg extracts capable of promoting apoptosis were reconstituted by combining cytosolic and membrane fractions of *Xenopus* egg extract preparations. Normal mouse liver nuclei were added to a final concentration of 1×10^4 nuclei/μL. An ATP regeneration system was provided (10 mmol/L phosphocreatine, 2 mmol/L ATP, 0.17 mg/mL creatine phosphokinase). Control or experimental proteins were added to concentrations described for each experiment. Total reaction volume was kept constant within each experiment by diluting APC or control protein samples to the same volume with protein dialysis buffer. Assay components were mixed on ice and transferred to room temperature to initiate the apoptotic time course. Aliquots were removed at each of the indicated time points for evaluation of caspase-mediated lamin B cleavage. Aliquots were electrophoresed through 10% SDS-PAGE gels, blotted as described above, and probed with 750 ng/mL anti–lamin B antibody (C-20). Full-length lamin B (70 kDa) is cleaved by caspase-3 and caspase-6 into 25- and 45-kDa protein fragments; only the 45-kDa fragment of lamin B is recognized by anti–lamin B C-20. Additional bands on Western blots are due to cross-reactivity with proteins from the *Xenopus* egg extract and were verified by Western analysis of normal mouse liver nuclei in the absence of *Xenopus* egg extract. The time of apoptosis is defined by the time point at which the 45-kDa caspase-mediated lamin B cleavage product is 2-fold the intensity of background as measured by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA).

For measurement of apoptosis using colorimetric techniques, apoptotic extracts were reconstituted by combining cytosolic and membrane fractions of *Xenopus* egg extract preparations. Control or APC protein preparations were added to concentrations described for each experiment, mixed on ice, and transferred to room temperature to initiate the apoptotic time course. Aliquots were removed at each of the indicated time points and frozen on dry ice for evaluation of caspase-3 activity. Each 2.5 μL aliquot was incubated with 200 μL of 250 mmol/L sucrose, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 20 mmol/L HEPES-HCl (pH 7.5), and 1 mmol/L DTT. Z-DEVD-pNA (80 μmol/L, Biomol Research Labs, Inc., Plymouth Meeting, PA) were added immediately before use. The absorbance at 405 nm was measured immediately and again 2 hours later. The change in absorbance represents caspase-3 activity in the sample aliquot and was plotted against time in hours from the start of the time course. Two hours were selected empirically as the longest incubation period consistently within the linear range of Z-DEVD-pNA cleavage by the caspase-3 within each reaction aliquot. The time of apoptosis is defined as the time at which the increase in absorbance at 405 nm is half-maximal.

Immunodepletion of X. laevis *APC from Egg Extract*

Xenopus APC was immunodepleted from egg extract using a mixture of the anti-human APC antibodies APC-Ab1 and APC-Ab5. Cytosolic fractions of egg extracts were precleared by incubation with 100 ng/μL normal mouse IgG and Pansorbin cells (Calbiochem) for 30 minutes at 4°C while rotating. Pansorbin was pelleted by centrifugation at 8,000 × *g* for 6 minutes; fresh Pansorbin was added to the supernatant, incubated for 30 minutes at 4°C while rotating, and pelleted by centrifugation. Half of the supernatant was incubated with 50 ng/μL APC-Ab1 and 50 ng/μL APC-Ab5 and half was incubated with 100 ng/μL normal mouse IgG, as a control, for 30 minutes at 0°C. Three times the sample volume of Pansorbin were added to each sample followed by incubation for 30 minutes at 4°C while rotating. Pansorbin was pelleted by centrifugation at 8,000 × *g* for 6 minutes and the supernatants were subjected to a second round of depletion for 30 minutes at 0°C. Three times the sample volume of Pansorbin were added to each sample followed by incubation for 30 minutes at 4°C while rotating and pelleting by centrifugation at 8,000 × *g* for 6 minutes. The supernatants were incubated with fresh Pansorbin followed by incubation for 30 minutes at 4°C and pelleting by centrifugation. APC-depleted and mock-depleted extracts were snap frozen and stored at −80°C.

Western Analysis of X. laevis *Egg Extract*

Western analysis of *Xenopus* egg extracts were done by separating proteins by electrophoresis through 10% polyacrylamide gels and electroblotting onto Immobilon-P polyvinylidene difluoride membrane. Blots were blocked by immersion in 1× TBS [20 mmol/L Tris base, 137 mmol/L NaCl (pH 7.6), 5% nonfat dry milk] for 1 hour at room temperature and probed with 200 ng/mL anti-caspase-8 H-134 (Santa Cruz Biotechnology) or 200 ng/mL anti-caspase-9 H-83 (Santa Cruz Biotechnology) in 1× TBS (pH 7.6), 5% nonfat dry milk for 1 hour at room temperature. Blots were washed twice with 1× TBS, 0.1% bovine serum albumin and probed with 200 ng/mL peroxidase-labeled anti-rabbit secondary antibody (Vector

Laboratories, Burlingame, CA) in 1× TBS (pH 7.6), 5% nonfat dry milk for 1 hour at room temperature. Blots were washed four times with 1× TBS, 0.1% bovine serum albumin and developed using Renaissance chemiluminescence reagents.

Inhibition of Transcription and Caspase Activity

Transcription by RNA polymerase II was inhibited by addition of 2 ng/μL α-amanitin to extracts before the addition of exogenous test or control protein preparations. Z-LEHD-FMK (Calbiochem) was added to extracts at a final concentration of 2 or 10 nmol/L. Z-IETD-FMK (Calbiochem) was added to extracts at a final concentration of 2 or 10 nmol/L. These concentrations are 2× IC_{50} or 10× IC_{50} for Z-IETD-FMK in cell extracts (31).

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