Mitochondrial Targeting of Adenomatous Polyposis Coli Protein Is Stimulated by Truncating Cancer Mutations

REGULATION OF Bcl-2 AND IMPLICATIONS FOR CELL SURVIVAL

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The adenomatous polyposis coli (APC) protein tumor suppressor is mutated in the majority of colon cancers. Most APC gene mutations cause deletion of the C terminus and disrupt APC regulation of β-catenin turnover, microtubule dynamics, and chromosome segregation. Truncated APC mutant peptides may also gain unique properties, not exhibited by wild-type APC, which contribute to tumor cell survival and proliferation. Here we report a differential subcellular localization pattern for wild-type and mutant APC. A pool of APC truncation mutants was detected at mitochondria by cellular fractionation and confocal microscopy. In contrast, wild-type APC located poorly at mitochondria. Similar results were observed for endogenous and stably induced forms of APC, with the shortest N-terminal mutant peptides (N750, N853, N1309, N1337) displaying the strongest mitochondrial staining. The knock down of mutant APC(N1337) in SW480 tumor cells caused an increase in apoptosis and mitochondrial membrane permeability, and this correlated with reduced Bcl-2 protein levels in mitochondrial fractions. Interestingly, the silencing of APC did not alter expression of β-catenin or the apoptotic regulatory factors Bax, Bcl-xL, or survivin. APC formed a complex with Bcl-2 in mitochondrial fractions, and this may contribute to the APC-dependent regulation of Bcl-2. We propose that a subset of cancer mutations induce APC mitochondrial localization and that APC regulation of Bcl-2 at mitochondria may contribute to tumor cell survival.

Mutations in the adenomatous polyposis coli (APC) tumor suppressor gene contribute to the pathogenesis of the benign polyposy syndrome, familial adenomatous polyposis (FAP), and are responsible for the majority (>80%) of sporadic cases of colon cancer (1–3). Most APC gene mutations are detected within the central mutation cluster region (MCR) and are early events in the tumorigenic process. The mutations generate truncated APC peptides that lack a C terminus, causing loss of binding to several partners and to microtubules (4). APC is a large protein comprising 2843 amino acids, and interacts with proteins involved in the Wnt signaling pathway and cytoskeletal organization (5). Many APC mutations disrupt APC-dependent β-catenin turnover, causing oncogenic β-catenin to accumulate in the nucleus and activate transcription of genes that promote cell transformation, leading to tumor formation (1, 6). APC is multifunctional and localizes to several subcellular compartments (4). APC can translocate into and out of the nucleus (7), and in the cytoplasm APC accumulates at the ends of microtubule (MT) bundles in cortical clusters near the plasma membrane (8, 9). APC has also been detected at the mitotic spindle (10, 11), centrosomes (12), and at actin-dependent membrane regions (13, 14).

Truncating cancer mutations alter APC retention at microtubules and its movement to membrane clusters (9, 15), and disrupt several processes including regulation of β-catenin degradation and chromosome stability (3, 4, 6). APC mutants retain the ability to shuttle between nucleus and cytoplasm (16, 17), and to locate at centrosomes (12) and membrane contact sites (14); however, a unique subcellular distribution has not yet been ascribed to the truncated APC peptides. While the mutational loss of APC C-terminal sequences is regarded as integral to the initiation of colon cancer through loss of APC tumor suppressing functions, there is also evidence that APC truncation mutants exert certain dominant functions that might contribute to the tumor cell phenotype. For instance, APC mutants were shown to stimulate ASEF-dependent cell migration (18), and more recently were reported to dominantly inhibit cytokinesis (19). In contrast to full-length APC, which is pro-apoptotic when overexpressed in colon cancer cells (20), there is also evidence suggesting a pro-survival function of APC mutants in colon tumor cell lines (21–23).

In this study, we show for the first time a preferential targeting of truncated mutant APC to a unique subcellular location: the mitochondria. We employed a range of complementary
methodologies including confocal microscopy and subcellular fractionation to demonstrate that unlike wild-type APC, which displays a very poor mitochondrial residency, a sub-set of truncated APC isoforms (mainly those whose sequence terminates prior to amino acid 1555) strongly localize to mitochondria. In SW480 colon cancer cells which express only a truncated form of APC (1–1338), the loss of mutant APC correlated with induction of apoptosis and also a loss of the anti-apoptotic factor Bcl-2 from mitochondria. We propose that the differential targeting of mutant APC to mitochondria promotes the localized accumulation of Bcl-2, and that this provides a novel and unexpected pathway contributing to tumor cell survival.

**MATERIALS AND METHODS**

**Cell Culture, Transfections, and Treatment**—Human SW480 (APC protein truncated at amino acid 1337, referred to as N1337), HT-29 (APC protein truncated at amino acids 853 and 1555, referred to as N853, N1555), HCT 116 (full-length APC) colon carcinoma cells, and U2OS osteosarcoma (full-length APC) cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. To carry out transient transfections, the cells were seeded at 75% confluence and treated for 4 h and then the medium was removed and APC knock down was preceded by GFP-Bcl-2 transient transfection, first the plasmid was transfected using FuGENE HD reagent as instructed by the supplier (Roche Applied Science.), then processed 48-h later. The nonsteroidal anti-inflammatory drug sulindac sulfide (Sigma) was used at 200 μM.

**Plasmids**—The pCMV-APC plasmids used in this study were kindly supplied by Bert Vogelstein. The GFP-tagged forms of APC including APC-(1–302), APC-(334–900), APC-(1941–2002), and APC-(2650–2843) were previously described (9). The plasmid pGFP-APC-(1379–2080) was generously provided by Dr. Richard Youle (25). pGFP-Bcl-2 was kindly supplied by Bert Vogelstein. The GFP-tagged forms of four APC isoforms (mainly those whose sequence terminates prior to amino acid 1555, referred to as N853, N1555, H11002, and H11003) were stained with antibodies against cytochrome c or with Mitotracker Red dye CMXRos (Molecular Probes). Dilutions used were: APC monoclonal antibody Ab7 (1:100, Oncogene Research), unpurified rabbit polyclonal anti-M-APC, corresponding to residues 1034–2130 (1:4000), (8); anti-Myc tag (1:500, Santa Cruz Biotechnology); anti-cytochrome c (1:100, BD Pharmingen). The following secondary antibodies were used: Alexa-488 or Alexa-594-conjugated donkey or goat antibodies to mouse or rabbit immunoglobulin G (Molecular Probes). In all cases, DNA was stained using Hoechst 33258 (1:500). Coverslips were mounted with Vectorshield aqueous mountant (Vector Laboratories) and observed and photographed using an Olympus BL51 fluorescence microscope at ×400 magnification. A SPOT32 camera was used for general image capture. Cell images for deconvolution were collected using a Zeiss Axiosvert 200 inverted microscope with the appropriate bandpass filters. The objectives used were a LD-Plan Neofluar ×40 or a Plan Apochromat Neofluor ×63. Z-stack images were collected at incremental steps of 1 μm. The images were collected for each cell and resolved using iterative deconvolution analysis (AxioVision Rel. 4.5 Software). The three-dimensional image analysis was performed after image capture with an Olympus Fluoview FV1000 confocal microscope. The objective used was UPLSAPO 60XW, and z-stacks of 0.2 micron step-size were captured, then analyzed using ImageJ software.

**Subcellular Fractionation Analysis**—Colon cancer cells and inducible cell lines were separated into mitochondrial and cytoplasmic fractions using the Q-proteome Mitochondria Isolation kit (Qiagen). After washing, cells were suspended in Lysis Buffer, which selectively disrupts the plasma membrane without solubilizing it, resulting in the isolation of cytosolic proteins. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and the endoplasmic reticulum, remained intact and were pelleted by centrifugation at 1000 × g for 10 min. The resulting pellet was suspended in Disruption Buffer, repeatedly passed through a narrow-gauge needle (26 or 21 gauge), and re-centrifuged to pellet nuclei, cell debris, and unbroken cells at 1000 × g for 10 min. The supernatant which contains mitochondria and the microsomal fraction was re-centrifuged to pellet mitochondria at 6000 × g for 10 min. After removal of the supernatant, mitochondria were dissolved using a lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40). A protease inhibitor solution was added (Roche
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Diagonstic. The same buffer was used to obtain total cell extracts.

For high purity preparations, the mitochondria pellet was resuspended in Mitochondria Purification Buffer and carefully pipetted on top of layers of Purification Buffer and Disruption Buffer (density cushion). The band formed in the interface of both buffers was removed and pelleted in Mitochondria storage buffer.

Immunoprecipitation and Immunoblot Analysis—The lysis was obtained by subcellular fractionation of lystate from total proteins centrifuged at 15,000 × g for 10 min at 4 °C. An aliquot of each supernatant was taken to determine the concentration of protein using Bradford solution. 1 mg of protein was incubated with 2 μg of APC antibody (Ab5, Oncogene Research) or normal mouse IgG (N103, Oncogene Research) overnight at 4 °C with continuous end-over-end mixing. Next, 40 μl of Protein A-Sepharose CL4B (GE Healthcare Bio-Sciences) was added and incubated at 4 °C for 2 h with continuous end-over-end mixing. The Protein A Sepharose immunocomplexes were pelleted by centrifugation at 1000 × g for 1 min and the supernatant was discarded. The pellet was washed three times with 500 μl of wash buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40). Pellet was resuspended in 40 μl of 2X Laemmli sample buffer and heated at 100 °C for 10 min. The samples were separated and transferred as described in detail (17). The following antibodies were used for the detection: APC (Ab1, 1:100 dilution, Oncogene Research); Bcl-2, Bax, Bcl-xL (1:1000, Cell Signaling Technology); survivin (clone 60.11, 1:1000, Novus Biologicals, Inc); purified recombinant protein A/G, peroxidase-conjugated (1:15000, Pierce-Biotechnology); α-tubulin (1:1000, Sigma), cytochrome c (1:100, BD Pharmingen), Prohibitin (1:100, Abcam).

Detection of Apoptosis—(a) Flow cytometry. After treatment, attached and floating cells were harvested and fixed with 85% methanol for 2 h. Cells were pelleted and suspended in a hypotonic fluorescent solution including propidium iodide.

RESULTS

APC Cancer Mutants Display Enhanced Localization at Mitochondria—We previously detected truncated mutant APC in the cytoplasm and the perinuclear zone of SW480 colon tumor cells (17), with a staining pattern reminiscent of mitochondrial distribution. To test whether the anti-apoptotic activity of APC(N1337) (encoding the first 1337 amino acids of APC) correlates with mitochondrial localization, we stained SW480 cells with antibodies against APC and the mitochondrial marker cytochrome c. As seen in immunofluorescence microscopy images using antibody Ab7, APC did co-localize with the mitochondria (Fig. 1A). Similar co-staining was observed with the mitochondria-specific dye, Mitotracker (data not shown). Moreover, mitochondrial co-staining of endogenous mutant APC and cytochrome c was detected with anti-APC antibody M-APC, and the specificity of this staining pattern was verified by silencing of APC by RNAi, which caused a loss of staining at mitochondria (Fig. 1B). The mitochondrial staining pattern for APC was also evident in transverse cross-sections of SW480 cells imaged by deconvolution microscopy (Fig. 1C). We noted that full-length APC displayed some co-staining with mitochondria (supplemental Fig. S1), but in general was not as pronounced as mutant APC.

APC Mutants Are Detectable in Mitochondrial Cell Fractions—APC was detected predominantly in the cytoplasmic fractions of SW480 cells by Western blotting using antibody Ab1 (Fig. 2A, left panel). To confirm the endogenous APC staining pattern, we used an antibody to the oncoprotein p53, which is known to localize to the mitochondria (18). As seen in Fig. 2A, right panel, p53 co-localized with mitochondria, suggesting that the staining pattern observed for APC is consistent with its known subcellular localization. Note that the intensity of staining observed for APC in mitochondria is much weaker than that observed for p53, consistent with our previous observation that truncated APC is less stable than full-length APC (17).

FIGURE 1. Detection of APC at mitochondria by immunofluorescence microscopy. A, APC mutant N1337 (amino acids 1–1337) was detected in SW480 cells using anti-APC monoclonal antibody Ab7 (1:100, Oncogene Research), stained green with secondary AlexaFluor 495 antibody (Molecular Probes). Mitochondria were detected with cytochrome c antibody (1:200, Pharmingen, stained red). Cells were analyzed by fluorescence microscopy with representative images shown. B, SW480 cells were stained M-APC antibody (1:4000 dilution), mitochondria detected with cytochrome c antibody and nuclei marked with Hoechst dye. Cells were transfected with control or APC-specific siRNA as described under "Materials and Methods," and reveal specific staining of mitochondria. The images were processed under identical conditions to enable an unbiased comparison. C, staining of APC in SW480 cells and analysis by confocal microscopy (arrows highlight mitochondria staining). The green spot of APC is the centrosome.
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A

**FIGURE 2. Detection of endogenous APC at mitochondria by Western blot analysis.** A, SW480 cells were fractionated into nuclear (Nuc), cytoplasmic (Cyt), or mitochondrial (Mito) fractions as described under "Materials and Methods." Total (60 μg) and fractionated extracts (each 80 μg of protein) were separated by SDS-PAGE and analyzed by Western blot, detecting APC with Ab1 antibody. Antibodies were used to detect markers of cytosol (α-tubulin), mitochondria (cytochrome c), nucleus (Topoisomerase II), and nucleus/cytosol (PCNA, proliferating cell nuclear antigen). B, the cell lines HCT116 and U2OS (full-length APC) and HT29 (mutant APC N1555 and N853) were also fractionated and analyzed by Western blot. Prohibitin antibody was used to verify integrity of the mitochondrial extract. C, a larger scale purification of HT29 cell mitochondrial and cytosolic fractions was performed and APC N853 and N1555 were immunoprecipitated by Ab5, then detected after Western blotting by Ab1 antibody. This two-step approach, using IgG as a negative control, revealed detection of both APC mutants in cytosol, but only the smaller N853 mutant in the mitochondrial fraction.

Imaging data, mitochondrial extracts were enriched from fractionated cells and analyzed for endogenous APC staining patterns. As shown in Fig. 2A (right panel), the APC (N1337) mutant was clearly detectable in the mitochondrial fraction. In addition we detected a smaller ~90-kDa cleavage product (26), which was exclusively observed in mitochondria (Fig. 2A). The fractionation approach was optimized to reduce contamination from the cytoplasm (using α-tubulin antibody as marker) and the nucleus (using PCNA antibody as marker), while the mitochondrial enrichment was verified using cytochrome c antibody (Fig. 2A). We then tested HT29 colon cancer cells, which express two different truncated APC mutants encoding the first 853 or 1555 amino acids. Intriguingly, only the shorter APC mutant (N853) was detectable in mitochondrial fractions; the APC(N1555) mutant localized poorly at mitochondria (Fig. 2B, center blot). To confirm this finding, APC was immunoprecipitated from HT29 cell fractions with an antibody recognizing the N terminus (Ab5) and then Western blots probed with a different antibody (Ab1); the IP experiment also showed differential targeting of the shorter APC mutant (Fig. 2C). These data revealed mitochondrial accumulation of specific truncated APC mutants smaller than 1555 amino acids.

Cell lines expressing full-length APC were also examined for APC at mitochondria; however, in contrast to the shorter mutant peptides, wild-type APC was expressed poorly in mitochondrial fractions from HCT116 colon cancer cells and U2OS osteosarcoma cells (Fig. 2B).

**Mitochondrial Targeting of Mutant APC Detected in APC-inducible Cell Lines**—To further validate the above result, we induced stably transfected HEK293 cells to compare mitochondrial staining of inducible full-length APC and C-term deletions that correspond to known mutations (N1807, N1309, N750). Full-length Myc-tagged APC displayed a partial and weak co-localization with cytochrome c at mitochondria after 16 h of induction with tetracycline (Fig. 3A, bottom row). However, the mutant APC (N1309), very similar in size to APC (N1337) expressed in SW480 cells, showed a stronger and unmistakable recruitment to mitochondria (Fig. 3A). The N1309 mutation is by far the most frequently occurring APC gene mutation detected in human colon cancers (2). The other APC mutants tested also displayed co-localization with mitochondria by deconvolution microscopy, and confirmed by confocal microscopy and three-dimensional image reconstruction (Fig. 3B and supplemental Fig. S2). In the absence of induction, the anti-Myc antibody gave only a weak background staining, and no clear co-localization with cytochrome c (Fig. 3A).

Next, we fractionated extracts from the APC-inducible cell lines and analyzed them by Western blot. The full-length form of APC was less well expressed after induction, and like the N1807 mutant, it displayed only weak mitochondrial detection (Fig. 4A). The N1309 and N750 mutants displayed stronger detection at mitochondria (Fig. 4A). The mitochondrial targeting of mutant APC was not dependent on β-catenin, in that the APC(N750) sequence does not bind to β-catenin. To confirm our results we used more refined mitochondrial preparations isolated by use of a density cushion (Fig. 4B), and observed APC in mitochondrial preparations free of α-tubulin and PCNA. We note that a closer examination of the blots for full-length Myc-tagged APC revealed some smaller degradation products; these constituted a minor pool of the total APC, but accumulated significantly in the mitochondria-enriched fractions (see Fig. 4C). A similar result was seen for Western blots of the ectopic N1807 mutant, possibly explaining its detection in mitochondria by fluorescence microscopy (Fig. 3). We did not observe significant degradation of other proteins in the mitochondrial extracts, and there was not extensive degradation observed for endogenous APC (Fig. 2). This observation is relevant because...
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A

HEK293 APC-inducible cell lines

myc-APC  cytoC  merge  uninduced

N750

N1309

N1807

APC full-length

B

3-D image zoom

APC(N750)  cyto C  overlay

FIGURE 3. Mitochondrial localization of APC in inducible cell lines. A, stable APC-inducible HEK293 cells (22) were induced with tetracycline and stained for Myc-tagged APC, endogenous cytochrome c and nuclei (Hoechst dye, blue). Full-length APC partially co-localized with mitochondria and co-staining increased with C-terminal deletion of APC. No co-staining was seen without induction. Cell images were acquired by Zeiss Axiovert 200 M microscope and resolved by deconvolution analysis using Zeiss Axiovision software. B, confocal three-dimensional co-localization of c-Myc-APC(N750) and cytochrome c, images were captured using an Olympus FV1000 confocal microscope and images processed with Image J software (see supplemental Fig. S2 for full details).

it suggests that APC cleavage products detected with an antibody that recognizes N-terminal fragments, similar in size to the truncation mutants, accumulate at mitochondria. We conclude that APC localizes at mitochondria and that this staining pattern is enhanced by truncating mutations of APC protein, in particular those smaller than 1400 amino acids in size.

Mapping the APC Mitochondrial Targeting Region to the Arm Domain—A computer-assisted search of the APC sequence did not identify a classic mitochondrial targeting signal (27). Therefore a series of GFP-tagged APC sequences that span the coding region of APC were transiently expressed in U2OS cells and the localization patterns then compared by microscopy and by fractionation/Western blot (Fig. 5). Consistent with the differential staining pattern observed for the N-terminal APC mutants, none of the C-terminal sequences (APC 1379–2080, 1941–2228, or 2650–2843) displayed significant mitochondrial localization by Western blotting, despite their strong cytoplasmic expression (Fig. 5A). Similar results were found by microscopic imaging (Fig. 5B and data not shown). This is an important finding, as it also eliminates involvement of several key binding partners (e.g. β-catenin, Axin, EB1, and DLG-1) in APC mitochondrial recruitment. In contrast, two different N-terminal sequences located to mitochondria. The APC-(1–302) sequence displayed a modest mitochondrial detection relative to the cytosol, whereas the Arm domain sequence (334–900) localized strongly at mitochondria (Fig. 5, A and B). The ratio of mitochondrial/cytosolic expression for APC-(334–900) was consistently similar to that observed for the shorter APC cancer mutants (Figs. 2 and 4), and implicates its involvement in targeting APC to mitochondria. The Arm domain is highly conserved and retained in most APC cancer mutants.

APC Truncation Mutants Protect SW480 Cells against Apoptosis and Loss of Mitochondrial Membrane Potential—The APC mutant (N1337) expressed in SW480 colon cancer cells was previously shown to be resistant to caspase-mediated cleavage and to help protect against apoptosis when overexpressed (28). The novel mitochondrial localization of this APC mutant has implications for its potential anti-apoptotic role. To study the potential anti-apoptotic function of human APC we tested the influence of transient transfection of APC mutants on resistance to apoptosis. APC constructs (full-length and mutants N932 and N1309) were transfected into SW480 colon tumor cells and the cells then treated with the drug sulindac sulfide, a chemopreventive agent used in colon cancer treatment. As shown in Fig. 6A, sulindac treatment induced apoptosis in SW480 cells as previously described (29). Transient expression of full-length APC did not reduce sulindac-induced apoptosis, and APC was pro-apoptotic in the absence of sulindac (Fig. 6A), as previously reported (20, 23). In contrast, overexpression of APC mutants N932 and N1309 did not induce apoptosis, and they effectively reduced apoptosis induced by...
suindac (see graph in Fig. 6A). Thus, in line with recent findings on the transcription-independent apoptotic action of APC (28), mutant forms including those which do not bind β-catenin (N932) are less apoptotic than wild-type APC and can protect against drug-induced apoptosis. Similar results were observed after staining cells with the mitotracker dye CMX-ROX, a marker of mitochondrial membrane potential (data not shown).

We then silenced endogenous APC(N1337) in SW480 cells by RNA interference and evaluated cells for apoptosis and loss of mitochondrial membrane potential (see Fig. 6B). The loss of endogenous APC was >80% efficient (see Fig. 6B, Western blot image) and produced a highly reproducible increase in apoptosis compared with cells transfected with RNAi control, as measured using flow cytometry and systematic scoring of abnormal apoptotic nuclei from Hoechst-stained cells (Fig. 6B, graphs, lower panel, and supplemental Fig. S3B). Furthermore, the knock down of APC elicited a comparable increase in cells with loss of mitochondrial membrane potential, as determined by scoring cells stained with the mitochondrial permeability marker, CMX-ROS (see images and graph in Fig. 6B and supplemental Fig. S3B). Thus, the loss of expression of APC(N1337) increased SW480 cell death.

APC Silencing Reduces the Level of Mitochondrial Bcl-2 in SW480 Cells—Does the mitochondrial targeting of APC mutants contribute to cell survival? To partly address this, we used APC siRNA to investigate the regulation of cell survival factors. In sporadic human colorectal cancer and in the base cells of FAP adenomatous crypts, the two primary anti-apoptotic factors Bcl-2 and survivin are overexpressed relative to the normal colonic mucosa (30). Both Bcl-2 and survivin locate at mitochondria where they inhibit apoptosis and promote tumorigenesis (30). We knocked down APC by siRNA and observed a ~60% reduction in total Bcl-2 protein levels compared with control cells (Fig. 7A, blots in left panel). A panel of different APC-specific siRNAs had a similar effect (see supplemental Fig. S3A). The result was highly selective in that loss of APC did not affect the expression of survivin, Bax, Bcl-XL, β-catenin, or actin (Fig. 7A, left panel). The siRNA-treated cells were then fractionated, and the loss of APC decreased Bcl-2 levels >5-fold more in the mitochondria than in cytosol or nucleus (Fig. 7A, right panel, and data not shown). These experiments were performed at least twice with similar results, and reveal for the first time that APC can regulate expression of Bcl-2 at mitochondria. The knock down of full-length APC in U2OS and HEK293T cells did not alter Bcl-2 levels, which were already much lower than that observed in SW480 cells (supplemental Fig. S4A).

Apoptosis—The possibility that mutant APC interacts with Bcl-2 was examined. Total extracts from SW480 or inducible HEK293 cells expressing APC(N1309) were immunoprecipitated with APC Ab5 monoclonal antibody and analyzed by immunoblotting. Endogenous Bcl-2 was captured by the APC antibody but not by control IgG beads (Fig. 7B, left panel). The interaction with APC was not observed for survivin or cytochrome c (data not shown). Further analysis revealed an association between APC and Bcl-2 in mitochondrial fractions (Fig. 7B, right panel, see arrows). Bcl-2 also bound to APC in the cytosol. The association between APC and Bcl-2 is not restricted to mutant APC, as immunoprecipitation of full-length APC in HCT116 cell extracts also captured Bcl-2 (Fig. 7C). Thus, while different forms of APC can complex with Bcl-2, it is the mutant form which will interact most with Bcl-2 at mitochondria. Consistent with the binding studies, GFP-tagged Bcl-2 co-localized with APC(N1309) at mitochondria by confocal microscopy (Fig. 8A). Similar co-staining was observed for endogenous mutant APC (Fig. 8A, bottom row). Our data strongly suggest that APC binds and stabilizes Bcl-2 at mitochondria (summarized in Fig. 8C), although additional mechanisms including indirect transcriptional influences on Bcl-2 expression cannot be excluded.

Apoptosis Caused by Knock Down of APC Is Blocked by Bcl-2 Overexpression—To determine whether the apoptosis caused by APC siRNA (see Fig. 6B) resulted from the loss of Bcl-2, we overexpressed Bcl-2 in SW480 cells where APC expression was silenced, and measured apoptosis by single cell assay. The overexpression of GFP-Bcl-2 blocked the induction of apoptosis caused by APC down-regulation (Fig. 8B). These results suggest that loss of mutant APC triggers apoptosis by diminishing the level of Bcl-2 at mitochondria. Conversely, we speculate that mutant APC at least partly contributes to tumor cell survival by...
promoting Bcl-2 accumulation at mitochondria and thereby rendering cells less sensitive to apoptosis.

**DISCUSSION**

Recent studies have suggested that truncated APC mutants can exert a dominant effect on tumor cell survival by reducing apoptosis (23, 28) or by affecting cell proliferation and genetic instability through changes in the mitotic spindle checkpoint (22). Here we report that mutations within the APC mutation cluster region stimulate movement of truncated APC peptides to mitochondria. APC cancer mutants lose the ability to bind directly to microtubules and certain binding partners (2, 6, 31), and the changes in protein binding or folding could stimulate the Arm-driven movement of an APC pool to mitochondria. We discovered that APC associates with Bcl-2 in cells, and that mutant APC is complexed with Bcl-2 at mitochondria and regulates its accumulation in that compartment. Bcl-2 is known to locate at the outer membrane of mitochondria (32), inferring a similar localization pattern for APC. The link between APC and Bcl-2 is underscored by our observation that APC did not bind or regulate survivin, another mitochondrial anti-apoptotic factor known to be overexpressed in colon tumors (30). We propose that truncated APC contributes to tumor cell survival by recruiting/stabilizing Bcl-2 at mitochondria. Thus, elevated levels of mutant APC and Bcl-2, which occur frequently in colon cancers, may in some cases contribute to tumor progression, implicating the disruption of this pathway as a potential anti-cancer strategy.

The subcellular localization of APC has long been a contentious issue (7, 17, 31, 33). This is the first report to identify a localization pattern specific to a subset of APC mutants and poorly displayed by wild-type APC (summarized in supplemental Fig. S5). A combination of microscopic image analysis and biochemical assays were used to demonstrate that APC mutant peptides terminating within the first 1337 amino acids exhibit mitochondrial localization. Longer APC sequences located poorly at mitochondria. The APC(N1337) sequence expressed in SW480 cells comprises the Arm domain, in addition to the three 15 amino acid repeats and one 20 amino acid repeat involved in binding to β-catenin. The slightly longer APC(N1555) sequence containing two additional 20 amino acid repeats did...
not locate at mitochondria (Fig. 2). In human colon cancers there is a strong genetic selection driving APC mutations that terminate around amino acid 1309 (1, 2, 4). There are several possible explanations for why this selection occurs, including the so-called "just-right" signaling hypothesis that proposes the APC sequences selected are of sufficient length to bind and partially regulate α-catenin, but are incapable of promoting its degradation (2). More recently, the APC(N1309) mutant was shown to be more resistant to caspase cleavage than other truncated forms of APC (28), raising the issue of conformational changes and the possibility that such mutations may enhance the accessibility of sequences that target APC to mitochondria.

The mitochondrial localization of APC was mapped to the N-terminal Arm domain, and to a lesser extent the sequence 1–302, thereby excluding any requirement for β-catenin and other C-terminal partners as mitochondrial chaperones for APC. The Arm domain is well conserved in different species and is present in most APC cancer mutants, comprising a series of seven armadillo-like repeats (reviewed in Ref. 1). The APC Arm sequence is known to bind at least four distinct proteins, each of which can influence APC localization to different subcellular regions: the guanine nucleotide exchange factor ASEF and the actin-cross-linking factor IQGAP1 can increase APC at membrane ruffles (13, 18), the protein phosphatase 2A subunit B56 can promote APC nuclear accumulation (16), and the kinesin adaptor protein KAP3A can drive APC along microtubules to membrane clusters (34). The Arm domain may therefore act as a flexible APC targeting signal depending on its accessibility and the Arm-protein interactions that dominate.

The specific protein-protein interactions involved in recruiting APC mutants to the mitochondria are yet to be determined. The mechanism by which certain truncated forms of APC move to mitochondria awaits determination. One of the most plausible viewpoints is that the majority of APC isoforms,
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including full-length APC, will be directed to mitochondria in the absence of other directional stimuli. For instance, in the case of APC mutants larger than 1555 amino acids (supplementary Fig. S5), the presence of microtubule binding sites and specific protein interaction sites for partners like Axin may drive APC into complexes or cytoskeletal structures that immobilize the protein in the cytoplasm. Alternatively, it is possible that certain C-terminal sequences interfere with Arm-directed mitochondrial localization. This scenario could potentially arise due to the reported interaction between the N and C terminus of APC (35), although it is perhaps less likely given that both truncated and full-length APC were found to associate with Bcl-2 in immunoprecipitation assays (Fig. 7). Alternatively, since the truncated forms of APC display increased nuclear-cytoplasmic shuttling compared with full-length APC (17), the mutant forms are more likely to contact a nuclear factor, which could potentially chaperone their mitochondrial transit.

What are the implications of APC accumulation at mitochondria? There are a spectrum of possibilities, including effects on oxidative metabolism and energy production, mitochondrial distribution and regulation of apoptosis. In this study we focused on the latter premise, that movement of APC mutants to mitochondria is linked to survival of tumor cells. The siRNA-mediated knock down of mutant APC(N1337) was found to cause an increase in apoptosis, and this correlated with a specific loss of Bcl-2 at mitochondria in SW480 cells. Moreover, transient expression of Bcl-2 blocked this apoptosis, implicating a link between APC and Bcl-2 in cell survival. This is the first demonstration that APC can regulate Bcl-2 expression, at least in SW480 cells. While a transcriptional regulation of Bcl-2 could possibly account for the effect, it is intriguing to note that both endogenous and ectopic mutant forms of APC associated with Bcl-2, consistent with formation of an APC protein complex that could stabilize Bcl-2 at mitochondria. It is not yet clear whether APC binds directly to Bcl-2, or indirectly through assembly of a larger protein complex.

The role of APC in apoptosis remains a little ambiguous, and as with many APC activities that are impaired by mutation, it is often explained as an indirect consequence of the increased B-catenin transcriptional activity which results from APC mutation (21). While this may be true in some cases, there is also evidence for transcription- and B-catenin-independent mechanisms of APC-dependent apoptosis (23, 28, 36). APC is susceptible to cleavage by caspases during the early stages of apoptosis (26, 37), and is cleaved at aspartate 777 located at the end of the Arm domain (26). Qian et al. (28) proposed that generation of the Arm-containing cleavage products can actually accelerate the rate of apoptosis, although that conclusion was based on transient overexpression of APC sequences and the analysis of apoptotic markers rather than actual cell death. Whether the accumulation of cleaved APC-Arm sequences (similar to APC(N750) shown in Fig. 3 and 4) triggers or prevents apoptosis is at present unclear, especially since similar sequences were previously linked to cell survival following a prolonged mitotic arrest (22). In this study, we found a clear correlation between the anti-apoptotic activity of APC mutants and their localization at mitochondria in SW480 cells. While this cell survival activity might be partly attributed to modest regulation of B-catenin transcription activity (38), or to increased resistance of the mutant APC to proteolytic cleavage (28), our findings raise a new possibility. We suggest that in colon tumors there is a mutational selection that favors accumulation of truncated APC peptides at mitochondria, and that the ability of these APC mutants to bind and regulate Bcl-2 at mitochondria may contribute to the continued survival and proliferation of tumor cells.

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