The APC Gene Product Associates with Microtubules in Vivo and Promotes Their Assembly in Vitro

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Abstract

Defects in the APC gene occur frequently in patients with familial adenomatous polyposis coli and are associated with the progression of sporadic tumors of the colon and stomach. We examined the subcellular location of adenomatous polyposis coli (APC) protein resulting from transient expression of full length and partial APC complementary DNAs in epithelial cells. Immunofluorescent detection revealed an association of APC with cytoplasmic microtubules. Expression of partial complementary DNA constructs indicated that the carboxy-terminal region of the APC protein, typically deleted in cancers, is essential for this association. The same APC polypeptides that associated with microtubules in vivo also dramatically promoted their assembly in vitro. These results suggest that wild-type APC protein binds to and affects the assembly of microtubules, whereas the mutants identified in tumors have lost this activity.

Introduction

Familial adenomatous polyposis coli is an autosomal dominantly inherited disease that predisposes carriers to a high probability of colorectal carcinoma (1). Mutations in the APC2 gene have been consistently identified in the germline of familial adenomatous polyposis coli patients (2–4) as well as in a high percentage of sporadic colorectal (5) and gastric tumors (6). In most cases, these mutations disrupt the coding sequence in the 5' half of the APC open reading frame (5–7) and result in the production of gene products lacking substantial portions of their carboxy-terminal structure (8–10). Since very little is known about the function of the APC protein, the impact of these carboxy-terminal deletions on the putative tumor suppressor activity of APC is unclear. Recently, the APC protein was reported to associate with catenins (8, 11), molecules known to be involved in cell adhesion. However, it is not clear how this relates to the dysfunction of mutant APC, inasmuch as both the truncated mutant and wild-type APC proteins bind catenins. The consistent deletion of the carboxy-terminal region of APC protein observed in cancers suggests that this region of the protein is essential to its tumor suppressor activity. In this study, we have examined the subcellular localization of APC with the particular aim of assessing the consequences of carboxy-terminal deletions. We found that wild-type APC protein, as well as fragments containing its carboxy-terminal region, associated with cytoplasmic microtubules, but a mutant lacking this region did not. The same APC polypeptides that bound microtubules also promoted their assembly in vitro.

Materials and Methods

The cloning of a full length APC cDNA and the generation of cDNA fragments indicated as APCs 1, 2, 3, and 4 was carried out as described previously (8). One set of cDNA constructs was engineered to contain the Glu—Glu epitope tag (12) and then subcloned into the baculovirus transfer vector pAcC13 (13) for expression in Sf9 insect cells. The baculovirus-expressed APC proteins were purified from Sf9 cells by anti “Glu—Glu” affinity chromatography as described previously for purification of recombi-

Fig. 1. Expression of APC polypeptides and reactivity of APC-specific antisera. (A) Schematic representation of APC gene products. General features of full length APC protein, as reported previously (2), are shown at top and fragments encoded by APC constructs 1–5 are aligned below. Numbers, positions of the terminal amino acid; GG, position of Glu—Glu epitope tag; ±, locations where nontagged versions were also constructed. (B) Coomassie blue-stained gel of immunopurified recombinant APC proteins. Left ordinate, approximate molecular weights (X 103) of standard proteins (STD); (C) Western blot of lysates from Sf9 cells transiently expressing APC constructs 1–5 or transfected with plasmid vector only. Approximately 25 μg of total protein from each lysate were loaded. Blots were developed with either anti-APC-2 or anti-APC-3.

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The abbreviations used are: APC, adenomatous polyposis coli; cDNA, complementary DNA; PBS, phosphate-buffered saline.
nent GAP proteins (12). For mammalian cell expression, APC cDNAs were subcloned into the pcDNA/AMP vector (Invitrogen) and used for transient transfections of SW480 cells, a human colorectal cancer cell line (14), or 293 cells, an immortalized embryonic kidney cell line (15). Cells were seeded at subconfluence in 6-well plates with 5 x 10⁶ or 3.5 x 10⁷ cells/well, respectively. The following day, 293 cells were transfected by a modified calcium phosphate precipitation method (16) and SW480 cells by lipofection using Transfectam (Promega). At 48 h posttransfection, cells were analyzed by immunofluorescence or harvested for lysis.

The generation, affinity purification, and use of rabbit polyclonal antisera to APC fragments 2 and 3 have been described elsewhere (8). For immunocytochemical detection of APC protein, 293 and SW480 cells were transfected as described above, except four coverslips were placed in each well of a 6-well plate. At 48 h posttransfection the cells were washed in PBS and fixed in 0.5% glutaraldehyde. The fixed cells were incubated for 30 min at 37°C with affinity-purified APC antibodies at a final concentration of 4.6 µg/ml (anti-APC-2) or 6.5 µg/ml (anti-APC-3) and either anti-β-tubulin (Monoclonal 2.1; Sigma) used at 1:100 or anti-keratin (guinea pig polyclonal; Sigma) used at 1:20. All antibody dilutions were made in PBS containing 0.05% saponin and then incubated for 30 min at 37°C with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) for anti-APC, Texas red-conjugated goat anti-mouse IgG (Cappel) for anti-β-tubulin, or Texas red-conjugated goat anti-guinea pig IgG (Accurate Chemicals and Scientific Corp.) for anti-keratin. All secondary antibodies were used at a dilution of 1:32. Coverslips were washed with PBS containing 0.05% saponin and mounted using FITC-guard (Testog, Inc.) onto glass slides. Stained cells were visualized by fluorescence using a Zeiss Axiovert 100 TV microscope at 100×. Photographs were taken with a Nikon 6000 camera body using Kodak TriX-pan black and white print film. Exposure times ranged from 0.25 to 8.0 s.

For the in vitro assembly of rhodamine-conjugated tubulin, tubulin preparations were kindly provided by Dr. T. Mitchison and L. Belmont (University of California at San Francisco, San Francisco, CA). Rhodamine-conjugated tubulin was prepared as described by Hyman et al. (17) using purified bovine brain tubulin prepared by the temperature-cycling method. Ten µl of purified tubulin (30 mg/ml) and 6 µl of rhodamine-conjugated tubulin (15 mg/ml) were mixed and then diluted 1/12.5 into BRB80 buffer (80 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8/1 mM MgCl₂/1 mM [ethylenebis(oxyethyl)ethylenediaminetetraacetic acid]/1 mM dithiothreitol/1 mM GTP). Three µl of the indicated APC protein were added to 10 µl of the tubulin mixture and then incubated at 37°C for 10 min. One µl of 0.1% glutaraldehyde in BRB80/60% glycerol was added to stop the reaction and 2 µl were spotted onto a coverslip for visualization. Slides were viewed and photographed at ×100 as described above for immunofluorescence of whole cells.

Results and Discussion

Several cDNA constructs, coding for either full length APC or fragments representing distinct regions of the protein (Fig. 1A), were expressed in insect SF9 cells and the proteins were purified by immu-
To determine the subcellular localization of the APC protein we examined various epithelial cell lines by immunofluorescence using the APC-specific antibodies. However, the expression of endogenous APC was rather low (less than 0.01% of total protein, based on quantitative immunoblotting analysis) and we were not able to unambiguously identify the endogenous protein by immunofluorescence. We therefore transiently overexpressed the APC gene and identified recombinant cells by relative immunofluorescence. Transient expression of full length APC in the kidney 293 cell and the colorectal SW480 cell resulted in significant immunofluorescence of 0.5—5% of the total cell population, whereas transfection with vector alone did not produce any brightly staining cells. APC-2 antibodies decorated a filamentous structure in both types of cells (Fig. 2, A and B). That the

Fig. 3. Localization of APC protein to cytoplasmic microtubules. (A and C) immunofluorescent detection of APC protein expressed in 293 cells. (B and D) costaining of cells shown in A and C with anti-β tubulin. (E and F) SW480 cells transfected with APC-5 were incubated overnight in 10 μM nocodazole and then fixed and stained with anti-APC-2 (E) and antikeratin (F). Bar, 10 μm.
filamentous staining pattern was due to expressed APC protein was verified by costaining with an antibody specific to an epitope tag engineered at the amino terminus of the recombinant APC protein (Fig. 2, C and D). The filamentous pattern seen in recombinant cells was compared to actin, tubulin, and keratin staining patterns (data not shown) and found to most closely resemble tubulin staining. Costaining a cell expressing APC with both anti-APC and anti-tubulin revealed a strong similarity between the two patterns (Fig. 3, A and B). We also identified a recombinant cell in telophase and, again, found APC protein costaining with tubulin, particularly at the midbody separating the daughter cells (Fig. 3, C and D). Moreover, treatment of cells with nocodazole, a microtubule-disrupting agent, resulted in dissolution of the filamentous staining patterns seen with APC antibodies, whereas intermediate filaments were still detectable with anti-keratin (Fig. 3, E and F).

To determine the region of the APC protein involved in microtubule localization we expressed partial gene constructs in 293 cells. As demonstrated by the expression of APC-4 fragment, deletion of the amino-terminal region of APC protein did not affect its overall localization pattern (Fig. 4A). In fact, expression of a fragment containing only the carboxy-terminal 70 kDa of the APC protein (APC-3) resulted in a filamentous staining pattern resembling that observed with tubulin staining (Fig. 4, C and D). However, the fragment lacking carboxy-terminal structure (APC-1), as is the case for mutant APC proteins detected in cancer cells, failed to localize to microtubules and was found distributed in deposits throughout the cell cytoplasm (Fig. 4B). To ascertain whether APC protein affected the assembly of microtubules in vitro we performed polymerization assays using rhodamine-conjugated tubulin (17) followed by immunofluorescent detection of assembled structures. As a positive control, glycerol was added to 25% and found to promote the assembly of numerous unbranched microtubules (Fig. 5A), whereas with buffer alone, microtubules were not observed (data not shown). The addition of APC-1 or APC-2 protein fragments did not have any significant effect in this assay (Fig. 5, B and C). Addition of APC-3 or APC-4 polypeptides resulted in the assembly of very large multiply branched microtubule arrays (Fig. 5, D and E). The stoichiometry required for the formation of large structures observed with APC-3 and -4 was approximated at one APC molecule/20 tubulin subunits, although, at lower stoichiometry (one APC/100 tubulin monomers) a significant number of smaller polymers were still observed (data not shown). These results demonstrate that the same APC polypeptides that associated with microtubules in vivo had a pronounced effect on their assembly in vitro. Because the APC-3 fragment contains a basic sequence that could nonspecifically interact with tubulin and possibly account for the effects in this assay, we tested basic histone proteins and polylysine as control proteins.
INTERACTION OF THE APC PROTEIN WITH MICROTUBULES

Fig. 5. Effect of APC proteins on microtubule assembly in vitro. A mixture of purified tubulin and purified rhodamine-conjugated tubulin was incubated at a final concentration of 1.5 mg/ml for 10 min at 37°C with a final concentration of the following additions. (A) 25% glycerol. (B) 0.08 mg/ml APC-1. (C) 0.12 mg/ml APC-2. (D) 0.1 mg/ml APC-3. (E) 0.22 mg/ml APC-4. Bar, 10 μm.

When used at molar concentrations approximately 3 times greater than those for the APC-3 and APC-4 fragments, no significant effects could be observed in the assay (data not shown).

The immunocytochemical and biochemical data presented here suggest that APC protein can associate with microtubules. When expressed in vivo, APC protein clearly decorated microtubules; and when added to purified tubulin in vitro, it promoted the assembly of microtubule arrays. In the vast majority of tumors where APC defects have been identified, the pattern of mutations is consistent with the expression of gene products lacking carboxy-terminal structure (5—7). Our results demonstrate that this region of the APC protein mediates both microtubule association in vivo and the assembly of microtubules in vitro. This implies that the neoplastic or preneoplastic state associated with a defective APC gene may result, in part, from the inability of the APC protein to interact with the microtubule network. It is not clear how the potential loss of microtubule regulation could contribute to neoplasia; however, the assembly and reorganization of microtubules are intimately coordinated with cell division. It has been suggested that disassembly of the microtubule network may directly activate initiation of DNA synthesis (18). Moreover, the link between microtubule dynamics and growth control is also evident from the effects of antimitotic drugs such as taxol, which stabilize the microtubule network (19).

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References


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