Tumor-Associated NH2-Terminal Fragments Are the Most Stable Part of the Adenomatous Polyposis Coli Protein and Can Be Regulated by Interactions with COOH-Terminal Domains

Zhuoyu Li and Inke S. Näthke
Division of Cell and Developmental Biology, School of Life Sciences, WTB/MSI Complex, University of Dundee, Dundee, United Kingdom

Abstract
Truncation mutations in the adenomatous polyposis coli (APC) gene are responsible for familial and sporadic colorectal cancer. APC is a large, multifunctional protein involved in cell migration, proliferation, and differentiation. Dominant effects that have been attributed to the NH2-terminal fragments of APC expressed in tumors may result from loss of functions due to lack of COOH-terminal regions or gain of functions due to fewer regulatory interactions. Resolving this issue and determining how structural changes contribute to the multiple functions of the APC protein requires knowledge about the structural organization of the APC molecule. To this end, we used limited proteolysis to distinguish regions of the molecule with limited structure from those that form well-folded domains. We discovered that the NH2-terminal region of APC was most resistant to proteolytic degradation, whereas middle and COOH-terminal regions were significantly more sensitive. Binding of APC to microtubules protected COOH-terminal regions of APC against proteolysis, consistent with the idea that this region of the molecule becomes ordered when bound to microtubules. Furthermore, interactions between the NH2- and COOH-terminal domains of APC were identified in vitro and in vivo, suggesting that NH2-terminal fragments of APC may be regulated by interactions with COOH-terminal domains. Indeed, expressing COOH-terminal APC fragments in tumor cells resulted in changes in the protein interactions of endogenous NH2-terminal fragments in these cells. Thus, the dominant function of NH2-terminal APC fragments found in tumor cells could be explained by loss of this regulation in tumors where COOH-terminal domains are missing. (Cancer Res 2005; 65(12): 5195-204)

Introduction
Mutations in the adenomatous polyposis coli (APC) gene contribute to colorectal tumorigenesis and are commonly associated with sporadic colon cancers (1, 2). Germ line mutations in APC result in an autosomal dominantly inherited disease called familial adenomatous polyposis (1), that is characterized by the development of multiple colorectal adenomatous polyps that tend to progress to carcinomas if left untreated (3). The biological functions of the APC protein are diverse, complex, and not yet fully understood. APC is required for the Wnt-regulated degradation of β-catenin, an important regulator of transcriptional activation and cell adhesion (4–6), plays a role in chromosome segregation during mitosis (7–10), and is implicated in regulating cytoskeletal dynamics, in particular microtubules (11–15), but has also been described in filamentous actin (F-actin)-associated pools (16–18). Consistent with such diverse functions, a large number of binding partners have been identified for APC and the intracellular distribution of the APC protein is extremely complex: APC is associated with microtubules and F-actin and is also present in cytoplasmic and nuclear pools (19–23).

Little is known about the relationship between the diverse functions of APC. Some regions of the APC molecule mediate a number of distinct interactions raising the question how the functions of APC that result from these interaction are coordinated. For instance, the armadillo repeat–containing region in the NH2-terminal domain of APC binds to KAP3 to provide a link to kinesins and thus an indirectly link between APC and microtubules (24), it interacts with Asef to provide indirect links to F-actin (25), and it can bind to the B56 subunit of PP2A which is important for the scaffolding function of APC in the Wnt pathway (ref. 26; Fig. 1C). Thus, one region of APC alone seems involved in three distinct functions of APC. It is unlikely that all these interactions occur simultaneously raising the possibility that the interplay between intermolecular and intramolecular interactions is involved in targeting APC to a distinct function.

Mutations in APC lead to loss of COOH-terminal regions and the expression of NH2-terminal fragments of varying length. The idea that such NH2-terminal fragments have dominant functions is supported by recent reports showing that expression of NH2-terminal APC fragments leads to mitotic defects (9, 27). Whether such dominant effects of the NH2-terminal fragments of APC that are expressed in tumors are the result of loss of functions due to lack of COOH-terminal regions in such fragments or gain of functions due to fewer regulatory interactions is not known.

To begin to address the molecular organization of the APC molecule so that these questions can be explored in detail, we used limited proteolysis as a means to identify regions within the APC molecule that were particularly sensitive to proteases. The proteolytic fragments we most frequently recovered in these experiments were large NH2-terminal fragments that were similar to truncated fragments that result from the most common mutations in APC in cancer.

Our data further suggest that tumor-associated NH2-terminal fragments of APC can be regulated by associating with COOH-terminal regions and that the ability of APC to bind microtubules

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Inke S. Näthke, Division of Cell and Developmental Biology, School of Life Sciences, WTB/MSI Complex, University of Dundee, Dundee, DD1 5EH, United Kingdom. Phone: 01382-345821; Fax: 01382-345386; E-mail: inke@lifesci.dundee.ac.uk.

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efficiently is linked to this activity. We found that NH2- and COOH-terminal regions of APC bind to each other in vitro and when coexpressed in cells. Furthermore, introducing COOH-terminal APC fragments into tumor cells that normally only contain a truncated, NH2-terminal APC fragment (called "endogenous" APC protein), resulted in the recruitment of this NH2-terminal APC fragment to microtubules. In addition, under these circumstances, protein interactions of the endogenous NH2-terminal APC fragment changed. We propose that this interaction may be responsible for the regulation of functions done by the NH2-terminal domain in the context of the full-length protein. Thus, the dominant function of NH2-terminal APC fragments (9) could be at least partially explained by loss of such regulation in tumors as a result of truncation mutations.

Figure 1. Limited proteolysis of APC. A, APC was immunoprecipitated from cell lysates and digested with increasing amounts [0 (lane 0), 0.03 (lane 1), 0.06 (lane 2), 0.12 µg (lane 3)] of endopeptidase Asp-N, Lys-C, and chymotrypsin as indicated. Samples were either treated with buffers alone (ctrl) or with lambda phosphatase (dephospho) before digestion. Fragments were separated by SDS-PAGE transferred to nitrocellulose and detected using antibodies against the NH2-, middle (M), or COOH-terminal domain of APC. Distinct fragments are designated by numbers on the blot. The migration of molecular weight markers 220, 160, 120, and 100 kDa are indicated adjacent to each blot. B, schematic summary of the proteolytic fragments produced by the indicated proteases. Epitopes for the antibodies used to detect the fragments are indicated in vertically striped boxes above the sequence schematic of APC that shows the position of N-APC, M-APC, and C-APC fragments. Numbers next to each fragment correspond to the numbers in (A). Shaded or stippled regions indicate that a number of cleavage sites reside in this region. Arrows mark the approximate position of the cleavage sites and are shaded as indicated next to each protease name (right). C, output from the GlobPlot algorithm predicting the propensity for well-ordered structures. A downward slope indicates increased order; an upward slope represents increased disorder. Hatched bars indicate the position of recognized protein domains and mark the coiled coil domains (first two hatched bars before residue 400), armadillo repeats (residues 400-720), and two coiled coil domains at around 1580 and 1700). Please note that these last two are unique to human APC, whereas all others were detected in all APC sequences identified thus far. The estimated cleavage sites for the different proteases are indicated by arrows (top). The arrows are shaded to match the shading used to for each protease as marked in (B). The position of the armadillo repeats and the microtubule binding site are shown below the graph, as are some of the proteins known to interact with NH2-, middle-, or COOH-terminal regions of APC (below). Small grey boxes across the top of the graph show regions predicted to be particularly disordered.
Materials and Methods

Cell culture and transfection. HeLa, SW480, and DLD1 cells (respectively expressing wild type or truncated APC spanning residues 1-1309 and 1-1418) were routinely cultured at 37 °C in 5% CO2 in DMEM (Sigma, Poole, United Kingdom) with 10% fetal bovine serum. Cells were transfected using Polyfect 2000 (Invitrogen, Paisley, United Kingdom) for SW480 or Eugene 6 (Roche, Penzberg, Germany) for DLD1 cells using the protocol provided by the manufacturer. Insect cells, Sf21, containing recombinant baculovirus were cultured at room temperature in a spinner bottle in SF-900 II medium (Invitrogen) supplemented with 50 units/mL recombinant baculovirus were cultured at room temperature in a spinner bottle in SF-900 II medium (Invitrogen) supplemented with 50 units/mL recombinant baculovirus. Sf21 cells, stably expressing constructs encoding different APC fragments were grown in shaking cultures in HL5 medium at room temperature (28).

Plasmid constructs. Constructs for protein expression in human cell lines encoding for NH2-terminal (residues 1-1018 of human APC), middle-terminal (residues 1014-2038), and COOH-terminal (C-APC, residues 2038-2843; or C-APC-AS residues 2068-2843; C2-2, like C-APC but lacking residues 2168-2541; C2-1, like C-APC but lacking residues 2168-2390) fragments of APC, were cloned into pEGFP-C3 to introduce a green fluorescent protein (GFP) tag at the NH2 terminus of each fragment as described previously (29).

Baculovirus constructs using the pFB-Nhis10HA vector encoding the same APC fragments carried an NH2-terminal 10 His-HA-Flag tag but not GFP. For expression in Dictyostelium discoideum AX2 cells, these APC fragments were subcloned into the PB17SEYFP vector to introduce a YFP-tag at the COOH-terminal end of the APC fragments.

Antibodies. Antibodies against APC that were used for this study were mouse monoclonal anti-N-APC (ALi) reactive with residues 45 to 140 (as described for ab58 by abcam)1 mouse monoclonal anti-C-APC raised against an MBP fusion with the COOH-terminal region of human APC (as described for ab120, abcam)2 affinity purified rabbit polyclonal serum raised against and reactive with residues in the middle of human APC (11), rabbit polyclonal anti-N-APC antisera raised against residues 3 to 347 (30). Antibodies against Kap3 and B56α were obtained from Transduction Laboratories (Lexington, KY).

Limited proteolysis. HeLa cell lysates prepared in lysis buffer (50 mmol/L Tris, 100 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 40 mmol/L i-γ-glycerophosphate, 0.5% NP40) and corresponding to 1 mg of total protein were immunoprecipitated with protein G-agarose (20 μL), and antibodies (5 μg) directed against the NH2- or COOH-terminal domain of APC. Samples were split in half and either dephosphorylated with λ-phosphatase (New England Biolabs, Beverly, MA) or treated with control buffers. The agarose beads were then washed twice with lysis buffer and thrice with the protease buffer recommended by the manufacturer. Samples were then treated with a panel of proteases, including chymotrypsin, rabbit polyclonal anti-M-APC, or C-APC fragments fused to YFP were added to lysates from Sf21 cells containing the same fragments fused to HA. Concurrently, 40 μL of an anti-HA-antibody matrix (Roche) was preblocked with 0.4 mg/mL ovalbumin (Sigma) at 4 °C for 2 hours. The matrix was then washed with lysis buffer thrice before mixed cell lysates were added for 2 hours at 4 °C with rotation.

Immunofluorescence microscopy. Cells were seeded at 5 × 105 cells per well into 6-well plates containing collagen-coated coverslips (Sigma). Cells were allowed to adhere and grow for 24 hours before transfection with different APC constructs. Forty-eight hours after transfection, cells were fixed with –20 °C methanol for 5 minutes. The coverslips were treated with 4% donkey serum in PBS and 0.1% Triton X-100 for 1 hour. Endogenous, NH2-terminal APC fragments were detected with either a rabbit anti M-APC polyclonal antibody (diluted 1:500; ref. 11), or a polyclonal anti-N-APC antibody (1:1000; ref. 30) for 1 hour at room temperature. Tubulin was detected with a mouse monoclonal antibody (DM1a, Sigma-diluted 1:1000). After washing with PBS, 0.1% Triton, the appropriate fluorescently conjugated secondary antibodies were applied (diluted 1:150; Jackson ImmunoResearch, West Grove, PA) and DNA was counterstained with 4′,6-diamidino-2-phenylindole (Sigma). Fluorescence microscopy was done using a DeltaVision restoration microscope (Applied Precision, Issaquah, WA).

Immunoprecipitation. HeLa cells grown to 70% to 80% confluency on 15-cm dishes were washed twice with cold PBS and scraped into 1 mL of ice-cold lysis buffer containing a cocktail of protease inhibitors (10 μg/mL Leupeptin, Pepstatin A, and Chymostatin A), 50 mmol/L sodium fluoride, 100 μmol/L sodium orthovanadate. After 5 minutes incubation on ice, insoluble material was pelleted by centrifugation and discarded. 0.5 mg protein from supernatant was incubated at 4 °C for 2 hours with gentle agitation with 30 μL protein G-agarose to which 8 μg of specific primary antibody had been prebound. Precipitated complexes were washed thrice with lysis buffer. Finally, the complexes were resuspended in 80 μL SDS-PAGE loading buffer containing 5 mmol/L DTT and boiled for 5 minutes before application to SDS-PAGE gels.

Antibodies and Western blotting analysis. Immunoprecipitates (20 μL) or total cell lysate (50 μg) were applied to 4% to 12% SDS/polyacrylamide gels (Novex/Invitrogen, Carlsbad, CA), transferred to Protran membranes (0.1 μm pore size, Schleicher and Schuell, Dassel, Germany), and probed with primary antibodies as described (29). Primary antibodies were diluted in block solution: N-APC 1:1,000; M-APC 1:2,000; C-APC 1:1,000, GFP 1:1,000 (Roche). Detection was done with horseradish peroxidase–conjugated either anti-rabbit or anti-mouse antibodies followed by enhanced chemiluminescence (Pierce, Tottenhall, United Kingdom).

Results

Proteolysis of adenomatous polyposis coli protein reveals the NH2-terminal third as the most proteolysis-resistant portion of the molecule and shows that phosphorylation may alter its conformation. Because phosphorylation is important for some of the functions of APC and is known to occur in cells, we first set out to test whether phosphorylation altered the protease accessibility of residues within the APC protein. We compared the digestion pattern of phosphorylated

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1 http://www.abcam.com/?datasheet=128
2 http://www.abcam.com/index.html?datasheet=120

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and nonphosphorylated APC. Full-length APC was immunoprecipitated from HeLa cells with monoclonal antibodies to either the NH₂- or COOH-terminal region of APC. The proteolytic digestion pattern of untreated (phosphorylated, "ctrl") or dephosphorylated APC after treatment with a panel of proteases was then determined using domain-specific antibodies. The proteases we used included Asp-N, Lys-C, and chymotrypsin. We chose this panel of proteases because they have different specificities: Endopeptidase Asp-N cleaves at the NH₂-terminal side of aspartate and cysteic acid, chymotrypsin cleaves residues adjacent to large aromatic residues, and endopeptidase Lys-C cleaves COOH-terminal to lysine residues.

Figure 1 shows that the electrophoretic mobility of APC was visibly increased after phosphatase treatment confirming that APC is normally phosphorylated in cells. Phosphorylated APC was digested less efficiently by Asp-N and chymotrypsin but not Lys-C (Fig. 1A) as apparent by the presence of large fragments in control but not dephosphorylated samples.

The full-length APC protein can be divided into N, M, and C parts, each comprising approximately one third of the length of the protein; the N-APC fragment (residues 1-1000), contains the armadillo repeats, the M-APC region (residues 1000-2000) contains binding sites for β-catenin, GSK, and Axin, and the C-APC region (residues 2000-2843) contains binding sites for microtubules, EB1, and PDZ domains. Untreated, phosphorylated APC was digested into a number of specific fragments. Based on their estimated size and reactivity with antibodies to the three regions, we were able to assign the approximate region where cleavage had occurred. For example, Asp-N produced a large fragment of about 220 kDa that reacted with N-APC and M-APC antibodies (Fig. 1A and B, fragment 1) indicating that it was generated by cleavage within M-APC, near the boundary between M-APC and C-APC (Fig. 1B). Alternatively, a ~160 kDa fragment that only reacted with antibodies against M-APC and C-APC was also produced by this protease (Fig. 1A and B, fragment 3) and this fragment is likely to represent the complementary COOH-terminal portion. Increasing the amount of Asp-N resulted in the formation of an 80- to 90-kDa fragment that only reacted with antibodies against the NH₂ terminus of APC suggesting that it represents an NH₂-terminal fragment (Fig. 1A and B, fragment 2). Based on its size, it is likely that this fragment was the result of cleavage near the end of the armadillo repeats. Digestion with Lys-C produced a similar NH₂-terminal fragment (Fig. 1A and B, fragment 2*) suggesting that the end of the armadillo repeats constitutes a distinct boundary between domains and is more readily accessible than adjacent residues towards the NH₂ terminus. In addition, Lys-C produced a number of larger NH₂-terminally derived fragments of 110 to 160 kDa (Fig. 1A and B, fragment 4). Only the larger ones of these reacted slightly with the M-APC antibody suggesting that they, too, were NH₂-terminal fragments that resulted from cleavage near the junction between the N- and M-regions of APC.

Chymotrypsin also produced a number of similar NH₂-terminal fragments ranging from 90 to 130 kDa (Fig. 1A and B, fragment 4*). The amount of these NH₂-terminal fragments was much reduced when APC was dephosphorylated before digestion with chymotrypsin. These experiments revealed that the following regions of the APC molecule are unlikely to lie in well-ordered, globular structures but reside in regions with little structure or are on the exterior of the molecule: the end of the armadillo repeat region around residue 800, the beginning and middle of the M-APC region (residues 1000-1400) and around residue 1700.

One striking observation was that the NH₂-terminal region was most resistant to proteases (Fig. 1A) and relatively large fragments of about 90 to 160 kDa reactive with an antibody against this domain were found in digestions with all the proteases we used. Fragments that reacted with antibodies raised against the COOH-terminal domain could not be detected after any of the proteases treatments, suggesting this domain lacks extended regions of ordered structure and was easily digested (Fig. 1A). The middle region behaved similar to the COOH-terminal domain and smaller fragments derived exclusively from this region were rarely observed. To exclude the possibility that the antibody to which APC was bound during the proteolysis had a protective effect on the NH₂ terminus, we also used a COOH-terminally directed monoclonal antibody to isolate APC protein in parallel experiments. The digestion patterns obtained with the anti-COOH-terminal APC antibody was indistinguishable from those shown in Fig. 1A (data not shown) making it unlikely that binding to a specific antibody, affected the conformation of APC or provided protection to the specific regions near the antibody-binding site. Furthermore, the relative abundance of predicted proteolytic sites for the proteases used showed no correlation with the observed cleavage pattern. In fact, there are many predicted cleavage sites within the NH₂-terminal domain (Supplementary Material).

**Predicted structural domains and proteolysis-resistant domains coincide.** To examine how the position of cleavage sites correlated with other structural features of the APC molecule we applied the Globplot algorithm to the human APC sequence. This algorithm gives an overall view of probabilities for regions within proteins to be disordered or globular (31) and it also identifies known protein domains. A striking feature of the prediction was the high probability for ordered structure spanning the entire NH₂-terminal region as indicated by the long downward slope for this region of the molecule (Fig. 1C). Similar predictions were obtained using the sequences of all known APC proteins in other species (data not shown). This prediction strongly supported our observation that such NH₂-terminal domains of APC were stable towards proteases. The algorithm also predicted the presence of two coiled-coils in the middle and end of the M-APC region. However, this was specific for human APC and was not detected in APC sequences from other species (data not shown). One of the predicted cleavage sites for Asp-N fell between these two coiled-coils (Fig. 1C) consistent with the idea that the protease-accessible region lies between two well-ordered domains. The other estimated cleavage sites reside mostly in the middle of the molecule where only short stretches of well-ordered domains are predicted. Interestingly, the position of estimated cleavage sites in the middle of APC is similar to positions predicted to fall between small sections of order and disorder (Fig. 1C).

**Binding to microtubules protects the COOH-terminal part of adenomatous polyposis coli against proteases.** The structural analysis using GlobPlot also revealed that the direct microtubule binding site in the COOH-terminal third of APC (15), is likely to be completely disordered as indicated by the upward slope predicted for this section of the molecule (Fig. 1C). The previous finding that the structure of a purified protein fragment that represents this domain on its own was not altered significantly by heating to 70°C (15) is consistent with this prediction. We concluded that its
disordered state is one reason this domain is so susceptible to degradation by proteases, which led us to speculate that this particular domain may assume structure when bound to its ligand, microtubules. To test this hypothesis, we compared the digestion of a purified COOH-terminal fragment of APC, comprising residues 1034 to 2843 of human APC (previously called APC4, spanning M-APC and C-APC; ref. 29), in the presence of microtubules or an equal amount of unpolymerized tubulin. When bound to microtubules, this fragment was not digested significantly and could be recovered in the microtubule-associated fraction after protease treatment (Fig. 2). In the presence of an equal amount of unpolymerized tubulin, this fragment was almost completely degraded (Fig. 2).

Intramolecular interactions of adenomatous polyposis coli in vitro. Our limited proteolysis experiments revealed the NH2-terminal domain of APC as the region most resistant to proteases. On the other hand, the COOH-terminal domain of APC was easily digested by proteases but was significantly more resistant when bound to microtubules. Interestingly, phosphorylation decreases binding of APC to microtubules (29) but also increased its resistance to proteases (Fig. 1A). These data suggest that both phosphorylation and protein interactions play an important role in regulating the conformation and thus the function of specific domains within the APC protein. We wanted to establish whether intramolecular interactions could be part of this regulation. To measure interactions between different domains of APC, recombinantly expressed APC fragments were mixed and their interaction tested. We used two different systems to express epitope-tagged versions of N-APC, M-APC, and C-APC. Fragments tagged with YFP were expressed in D. discoideum and the same fragments tagged with HA were expressed in Sf21 cells using baculovirus. Using antibodies to the HA-tag, fragments from Sf21 cells were recruited to microtubules in the presence of C-APC as indicated by the finding that >90% of these proteins remained in the supernatant (Fig. 3D). However, when lysates from N-APC- and C-APC-expressing cells were mixed, there was a significant shift of N-APC into the microtubule-associated fraction consistent with our findings that N-APC and C-APC can bind to each other. M-APC was not recruited to microtubules in the presence of C-APC as indicated by the absence of the full-length M-APC fragment in the pelleted

**Figure 2.** Binding to microtubules renders APC more stable. A fragment of APC spanning residues 1014 to 2843, APC4, was incubated with unpolymerized tubulin (Tu) or Taxol-stabilized microtubules (Mt), using equivalent amounts of tubulin. Samples were then exposed to chymotrypsin for 15 minutes. Microtubules and associated proteins were isolated by sedimentation through a glycerol cushion. Sedimented and soluble material was subjected to PAGE. APC fragments were detected using a COOH-terminal antibody. Tubulin was visualized on a Coomassie gel (bottom). Lane 1, APC4 with unpolymerized tubulin (Tu) or microtubules (Mt) before digestion (using 1/30 of the entire sample). Lane 2, the same samples immediately after digestion with chymotrypsin for 15 minutes (using 1/3 of the entire sample). Lane 3, soluble material after sedimentation through the glycerol cushion (using 1/2 of the sample). Lane 4, pelleted material from the sedimentation (using 1/2 of the sample). No protease inhibitors were added during the centrifugation step and this caused continued proteolysis in the supernatant to result in little protein recovered in this fraction [compare the total before centrifugation (lane 2 Tu) and the supernatant after centrifugation (lane 3 Tu)].
microtubule-associated material (Fig. 3D). Importantly, the fraction of C-APC that bound to microtubules was not altered by the presence of N-APC. These results further support an association between NH2- and COOH-terminal APC domains that is not disrupted by the presence of microtubules and does not affect the ability of C-APC to bind microtubules.

The microtubule-binding region of C-APC is required for the interaction between C-APC and N-APC. To determine the residues involved in the interaction between the NH2- and COOH-terminal APC fragments, the interaction between the endogenous N-APC in SW480 cells and a number of C-APC fragments that differ in their ability to bind microtubules was determined (Fig. 4). Deleting residues up to residue 2067 (‘‘C1’’) of the major microtubule-binding site almost completely abolished binding of C-APC to N-APC (Fig. 4B), although similar amounts of all the C-APC fragments were expressed (Fig. 4A). These data suggest that the region rich in positively charged amino acids between residues 2220 to 2400 is involved not only in the interaction of APC with microtubules but is also necessary for the interaction between the NH2- and COOH-terminal domains of APC.

Recruitment of NH2-terminal adenomatous polyposis coli fragments in tumor cells to exogenous COOH-terminal fragments. To investigate whether interactions between COOH-terminal and NH2-terminal APC fragments could affect NH2-terminal fragment affinity for endogenous NH2-terminal APC fragments in tumor cells, we expressed C-APC in DLD1 cells. These cells only express truncated APC comprising residues 1 to 1418. The localization of the endogenous APC was compared in control and transfected cells. In untransfected cells or those transfected with GFP alone, the endogenous APC fragment was diffusely distributed throughout the cell (Fig. 5). Most notably, when cells were transfected with C-APC, the endogenous N-APC fragment was recruited to microtubules, which were decorated with C-APC. In contrast, when DLD1 cells expressing N-APC, middle-APC, or C-APC (C) were mixed with each other in the indicated combinations and then incubated with Taxol-stabilized microtubules, Microtubules and associated proteins were sedimented through a glycerol cushion and proteins in the pellet (P) and supernatant (S) were subjected to PAGE, transferred to nitrocellulose, and probed with an anti-GFP antibody. Microtubules and associated proteins were sedimented through a glycerol cushion and proteins in the pellet (P) and supernatant (S) were subjected to PAGE, transferred to nitrocellulose, and probed with an anti-GFP antibody. Microtubules and associated proteins were sedimented through a glycerol cushion and proteins in the pellet (P) and supernatant (S) were subjected to PAGE, transferred to nitrocellulose, and probed with an anti-GFP antibody. Microtubules and associated proteins were sedimented through a glycerol cushion and proteins in the pellet (P) and supernatant (S) were subjected to PAGE, transferred to nitrocellulose, and probed with an anti-GFP antibody. Microtubules and associated proteins were sedimented through a glycerol cushion and proteins in the pellet (P) and supernatant (S) were subjected to PAGE, transferred to nitrocellulose, and probed with an anti-GFP antibody.
Interaction between N-APC and C-APC causes changes in the protein interactions of N-APC. To determine how the binding of C-APC to N-APC affected protein interactions of N-APC, we investigated the pattern of proteins that could be immunoprecipitated with the NH2-terminal fragment in tumor cells in the absence and presence of C-APC. We found that the amount of Kap3 protein that could be detected bound to N-APC was significantly reduced in SW480 cells when they expressed C-APC compared with untransfected cells or those transfected with GFP, whereas the relative amount of B56α that bound to N-APC was increased, albeit slightly (Fig. 6). The total amount of any of these proteins did not change and the amount of N-APC that was immunoprecipitated was similar under all conditions (Fig. 6). These observations confirm that the interaction between C-APC and N-APC can indeed contribute to the regulation of the functions of the NH2-terminal domain.

Discussion

Our studies provide novel information about the structural organization and regulation of the APC molecule in cells. We obtained similar results using either immunoprecipitated full-length APC protein (Fig. 1) recovered with antibodies to either NH2- or COOH-terminal regions, or the largest APC fragment we can currently purify to homogeneity, which contains two thirds of the molecule, the middle and COOH-terminal regions of APC (Fig. 2).

We found that most of the NH2-terminal third of APC was resistant to proteases, whereas the middle and COOH-terminal regions were readily digested. This led us to conclude that the NH2 terminus of APC is well folded, whereas the middle and COOH-terminal regions are more disordered. The approximate mapping of one of the proteolytically sensitive sites to around residue 1700 (Fig. 1) was confirmed by the only successful attempt to obtain NH2-terminal sequences from proteolytic APC fragments. In this preliminary experiment, two fragments starting with residues between 1750 and 1950 were detected. Our inability to obtain NH2-terminal sequence information from many of the other fragments is consistent with the fact that the NH2 termini of many proteins are blocked and further supports our result that NH2-terminal fragments were most resistant to proteolysis.

These conclusions about the relative degree of organization of N-, middle-, and COOH-terminal regions of APC were strongly supported by predictions using GlobPlot, an algorithm that calculates the tendency within a protein for disorder and order/globularity (31).3 These predictions were consistent for the sequences of all known APC proteins, further supporting their validity.

In particular, these predictions revealed the microtubule-binding site as a region singularly unstructured, consistent with our observation that this part of the APC molecule is extremely sensitive to proteolytic digestion. This led us to hypothesize that the COOH-terminal domain, particularly the region around the microtubule-binding site, undergoes a significant conformational change when APC binds to microtubules. Indeed, binding of APC protein to microtubules but not unpolymerized tubulin protected it against proteolysis (Fig. 2).

We also found that phosphorylation rendered the NH2-terminal APC region more resistant to degradation suggesting that phosphorylation of APC affects the folding of the molecule so that in its dephosphorylated state, APC is more unfolded. An
alternative possibility is that proteins bound to APC were lost as a consequence of dephosphorylation. However, not all proteases we used were sensitive to the phosphorylation to the same degree even when they cleaved in a similar region suggesting that overall conformational changes or protein interactions were not affected by phosphorylation.

Our observation that cleavage readily occurred between residues 800 and 1300 was particularly interesting because this is the region of APC that is most commonly mutated in cancer (Fig. 3C). Indeed, the proteolytic fragments we detected correspond to fragments expected from the most common APC truncation mutations in cancers. These observations suggest that selection for APC mutations may correlate with the stability of the resulting fragment and may relate to the structural arrangements of this particular region.

Figure 5. Microtubule associated C-APC fragment recruits N-APC fragments that are expressed in tumor cells. DLD1 cells were transfected with GFP-C-APC or GFP. Cells were fixed and then stained with antibodies to detect the endogenous NH2-terminal APC fragment (B, F, K, O) and tubulin (C, G, L, P). GFP (I and N) or GFP-C-APC (A and E) were directly visualized. E-H and N-Q are enlargements of the regions indicated by the stippled boxes in (A-D) and (I-M). Bar, 15 \mu m (A-D and I-M) and 5 \mu m (E-H and N-Q). The increase in the endogenous N-APC observed in the cell in the middle that is transfected with higher amounts of C-APC was not always observed. Importantly, even in cells expressing low amounts of C-APC as shown in the enlargement (F), the redistribution of endogenous N-APC to microtubules was detected.

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4 http://perso.curie.fr/Thierry.Soussi/APC.html
The site of the initial mutation in the APC gene seems to dictate whether the "second hit" is another truncation mutation or whether the remaining wild-type APC allele is simply lost (34, 35). This is consistent with the idea that APC mutations that confer the necessary advantages to cancer cells are not only selected for loss of functions but that the balance between lost COOH-terminal function and retained NH2-terminal functions is also important (35). Indeed, we found that COOH-terminal fragments of APC bind to the NH2-terminal fragments. Based on this finding, we proposed that normally, in the full-length APC protein, different functions are carefully balanced and that the interaction between NH2- and COOH-terminal regions are an important feature of this regulation (Fig. 7; ref. 36). In our model, loss of the COOH-terminal region due to tumor-associated APC truncation mutations, results in loss of this regulation and disruption of this balance, which could produce a dominant effect of the remaining NH2-terminal domain that is expressed in tumor cells (Fig. 7).

The NH2-terminal domain of APC contains armadillo domains, which have been identified as the binding site for a number of other proteins (Fig. 1C). It is unlikely that all of these interactions can occur simultaneously and it is not clear which of these interactions may be most important for a dominant effect of NH2-terminal fragments in tumor cells. Importantly, our data suggest that interactions between NH2- and COOH-terminal domains may contribute to the regulation of the NH2-terminal domain. Most notably, the amount of Kap3 bound to NH2-terminal APC fragments in tumor cells was greatly reduced whereas the amount of B56a was slightly but reproducibly increased in cells also expressing C-APC. Although identical amounts of the endogenously expressed N-APC was recovered in all cases, the amount of Kap3 that could be detected bound to the N-APC fragment was greatly reduced whereas the amount of B56a was slightly but reproducibly increased in cells also expressing C-APC. The migration of molecular weight (kDa) markers is shown (left).

Figure 6. Changes in the protein interaction of N-APC in the presence of C-APC. A, equal amounts of protein from cell lysates from untransfected SW480 cells or those transfected with C-APC or GFP were prepared and subjected to PAGE and immunoblotted with antibodies against GFP, C-APC, Kap3, or B56a as indicated to reveal that the expression levels of these proteins was the same in all cells. The same lysates were immunoprecipitated with a monoclonal (B) or polyclonal (C) antibody against N-APC, and coprecipitated material was probed with antibodies against N-APC, Kap3, and B56a. Although identical amounts of the endogenously expressed N-APC was recovered in all cases, the amount of Kap3 that could be detected bound to the N-APC fragment was greatly reduced whereas the amount of B56a was slightly but reproducibly increased in cells also expressing C-APC. The migration of molecular weight (kDa) markers is shown (left).

Figure 7. Model of how truncation mutations in APC may result in loss of the balance between different functions. A, in full-length APC, functions carried out by different domains are balanced. The interaction between the NH2- and COOH-terminal domains of APC are tightly linked to the interaction of APC with microtubules and second that they contribute to the intricate balance that normally exist between direct and indirect interactions of APC with microtubules. One possibility is that the NH2-terminal APC fragments commonly expressed in tumor cells affect cell migration and that this activity is normally regulated by its binding to COOH-terminal

NH2- and COOH-terminal regions of APC, these data suggest the following: first, that regulatory interactions between the NH2- and COOH-terminal domains of APC are tightly linked to the interaction of APC with microtubules and second that they contribute to the intricate balance that normally exist between direct and indirect interactions of APC with microtubules.

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domains of APC in the full-length protein. However, testing whether cell migration is altered when COOH-terminal APC fragments are expressed in tumor cells with NH2-terminal APC to force this interaction is complicated by the ability of such COOH-terminal fragments to bind microtubules and increase their stability (29). Another possibility is that regulation of the NH2-terminal domain by the COOH-terminal domain of APC could be exerted by sequestering NH2-terminal fragments to specific sites like microtubules. This idea is supported by our result that recruitment of C-APC to microtubules in cells was dominant and was not affected by binding to N-APC. This is consistent with our in vitro data showing that binding of C-APC to microtubules was not reduced when N-APC is present, although the same domain mediates both of these interactions.

Our data also showed that the amount of B56α bound to N-APC in tumor cells was slightly but reproducibly increased when NH2-terminal fragments were bound to C-APC in cells. It is possible that this was simply a direct consequence of the loss of Kap3 which could have "made room" for B56α. Binding of B56α to APC is important for its role in Wnt signaling, whereas binding of APC to Kap3 is thought to play a role in the interaction of APC with microtubules and its targeting to microtubules ends. Although the increase in B56 bound to N-APC was small, these results raise the possibility that regulation of β-catenin and cytoskeletal organization by APC in tumor cells may be coordinated by interactions between different domains of APC.

In summary, we conclude that regulatory interactions between NH2- and COOH-terminal regions of APC contribute to normal APC function and that the loss of such regulatory interactions in tumor cells may contribute to the dominant effect of tumor-associated NH2-terminal fragments. Identifying the dominant effect that is most important to control in tumor cells expressing an "unrestrained" NH2-terminal APC fragments could provide the basis for the design of therapeutics that specifically target APC mutant cells.

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References


Tumor-Associated NH$_2$-Terminal Fragments Are the Most Stable Part of the Adenomatous Polyposis Coli Protein and Can Be Regulated by Interactions with COOH-Terminal Domains

Zhuoyu Li and Inke S. Näthke


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