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Advances in Brief

Association between Wild Type and Mutant *APC* Gene Products[1]

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Abstract

Germline mutations of the *APC* gene are responsible for familial adenomatous polyposis, an autosomal dominant inherited predisposition to colorectal tumors. Mutation of the *APC* gene is also an early, if not initiating, event for sporadic colorectal tumorigenesis. In both cases, almost all of the currently identified mutations of *APC* result in the truncation of the protein. In this study, we demonstrate that truncated APC proteins can associate with the wild type APC *in vivo*. Using *in vitro* expression and immunoprecipitation, we show that the first 171 residues of APC are sufficient for APC oligomerization and that the first 45 amino acids of APC is necessary for this interaction. These results indicate that most mutant APC proteins should be able to bind to wild type APC protein and perhaps inactivate it in a dominant negative manner.

Introduction

The *APC* gene was isolated by positional cloning from human chromosome 5q21 and has been implicated in FAP[3] as well as sporadic colorectal tumorigenesis (1–4). Thus far, there have been more than 60 different germline mutations of APC identified in FAP patients (5–11). A germline mutation of the murine homologue of *APC* produces a similar phenotype in mice (12, 13). *APC* is also somatically mutated in the majority of sporadic colorectal tumors, including very small benign tumors, suggesting that *APC* mutations initiate sporadic as well as inherited forms of colorectal neoplasia (2, 7, 14, 15). Almost all of the mutations of *APC*, both germline and somatic, result in the truncation of the gene product, through nonsense or frame-shift mutations. These truncated APC polypeptides could be nonfunctional gene products, or they could have a dominant negative activity upon the wild type APC, *e.g.*, by association with the wild type APC.

Analysis of the predicted amino acid sequence of APC showed that there were several regions with high probability of forming coiled-coil structures (1, 3). Such coiled-coil structures have been implicated in protein-protein interactions. Many of these regions are at the amino terminal end of APC and are preserved in all of the identified APC mutants. This raises the intriguing possibility that the amino terminal part of APC mediates its oligomerization and that mutant *APC* could associate with the wild type APC. To evaluate this issue, we examined the association of mutant and wild type APC proteins *in vivo* and *in vitro*.

Materials and Methods

Cell Lines. SW480 and HCT116 are human colorectal cancer cell lines (16, 17). 293 is an adenovirus transformed human embryo cell line (18). FAP 1211 and FAP 1309 are two lymphoblastoid cell lines derived from FAP patients harboring nonsense mutations of *APC* at codon 1211 or 1309, respectively (5, 19).

Monoclonal Antibody Production. A TrpE-APC fusion protein expression vector was constructed by cloning an *Xba*I fragment of *APC* containing codons 2537 to 2843 into the *Xba*I site of pATH3 (20). Production and isolation of the bacterially expressed fusion protein, immunization of mice, and production of monoclonal antibodies were performed as described (19).

Detection of APC Expressed *in Vivo*. Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5); 100 mM NaCl; 0.5% Nonidet P-40; 0.2% (w/v) 4-(2-aminoethyl)benzenesulfonylfluoride (Calbiochem); and 0.01 mg/ml each of chymostatin, leupeptin, antipain, and pepstatin A (all from Sigma). Cell lysates were precleared with normal mouse IgG and APC was immunoprecipitated with purified monoclonal antibodies. The immunoprecipitated proteins were visualized by Western blotting using monoclonal antibody FE9 as described (19). In mixing experiments, cell lysates were incubated together on ice for 1 h before immunoprecipitation.

Templates for *in Vitro* Transcription-Translation. HA-APC is a pBluescript (Stratagene) derivative with an insert coding for a HA epitope fused in frame to codons 1 to 1013 of *APC*. The template coding for APC-K (Fig. 3) is a pBluescript derivative containing codons 46 to 446 of *APC* with a translation initiation codon inserted 5′ to codon 46 via polymerase chain reaction. The template for *MCC* (21) has been described (22). All other templates were polymerase chain reaction products carrying a promoter for T7 RNA polymerase and a translation initiation signal at their 5′ ends. Fig. 3 diagrams the APC proteins encoded by these templates. Details of their construction are available upon request.

Detection of APC Expressed *in Vitro*. The TNT system (Promega) was used for coupled *in vitro* transcription-translation reactions. The reactions were carried out according to the manufacturer's instructions using T7 RNA polymerase and were stopped by adding cell lysis buffer described above to 250 μl. HA-APC and associated proteins were immunoprecipitated as described above with a monoclonal antibody against HA (Berkeley Antibody). Proteins were separated on sodium dodecyl sulfate-polyacrylamide gels and detected by fluorography.

Results and Discussion

We first evaluated monoclonal antibodies against the carboxyl terminus of APC by immunoprecipitation followed by immunoblotting. The DB1 antibody immunoprecipitated full length APC protein from HCT116 and 293 (Fig. 1), a result that was consistent with those obtained with antibodies against the amino terminus of APC (Ref. 19; data not shown). However, as expected, this monoclonal antibody did not immunoprecipitate APC from SW480, which expressed only an APC protein truncated at codon 1338 (2, 19). HG2, a second monoclonal antibody reactive with a different carboxyl terminal epitope, produced results similar to those obtained with DB1 (data not shown). The APC protein in SW480 was, however, efficiently immunoprecipitated by CF11, a monoclonal antibody specifically against the amino terminus of APC (Fig. 1).

We then used these monoclonal antibodies to determine whether truncated APC associated with wild type APC *in vivo*. Because most colon cancer cell lines that we have examined express only truncated APC (19), two lymphoblastoid cell lines derived from FAP patients

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; HA, hemagglutinin.

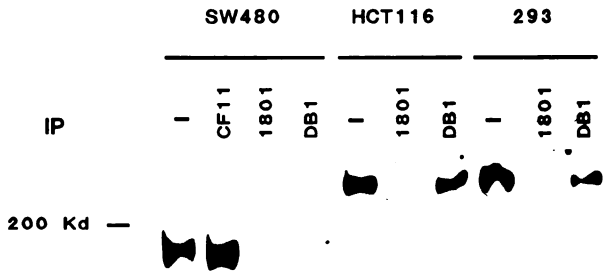

Fig. 1. Immunoprecipitation of APC with monoclonal antibodies. The SW480, HCT116, and 293 cell lines are described in the text. *IP*, monoclonal antibody used for immunoprecipitation. *Lanes* marked – indicate total lysate without prior immunoprecipitation. DB1 is a monoclonal antibody specific for the carboxyl terminus of APC. CF11 is a monoclonal antibody against the amino terminus of APC (19). PAb1801 (Oncogene Science) is a monoclonal antibody against p53 and was used as the negative control. *Kd*, molecular weight in thousands.

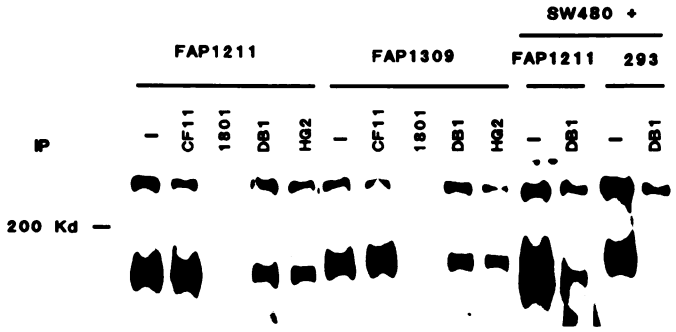

Fig. 2. Association of APC proteins *in vivo*. FAP1211 and FAP1309 are two lymphoblastoid cell lines derived from FAP patients described in the Materials and Methods. CF11, 1801, DB1, and – are described in legend to Fig. 1. HG2 is a second monoclonal antibody specific to the carboxyl terminus of APC.

that expressed both mutant APC (nonsense mutations at codon 1211 or 1309) and wild type APC were used for analysis. Because these carboxyl terminal antibodies recognized epitopes between codons 2537 and 2843, they could not directly immunoprecipitate the mutant APC proteins from these cell lines. Yet, in contrast to the result obtained from SW480, truncated APC was present in the immunoprecipitates when these lymphoblastoid cell lines were examined (Fig. 2). Moreover, although the truncated APC was apparently more abundant than the wild type APC in the cell lysates, the amount of mutant APC was the same as or less than that of the wild type APC in the immunoprecipitate from these lysates. These results strongly suggest that the truncated proteins associated with wild type APC *in vivo* and that only those mutant APC associated with wild type APC could be immunoprecipitated. When SW480 lysate was mixed with a lysate prepared from cell lines expressing full length APC prior to immunoprecipitation, the truncated APC from SW480 was not present in the immunoprecipitates (Fig. 2). This result argued against the possibility that the APC complexes present in the immunoprecipitates were formed after cell lysis.

In order to map the region responsible for APC homo-oligomerization, we used *in vitro* transcription-translation reactions. Because most of the regions predicted to form coiled-coil structures are within the amino terminal one-third of APC, we chose to examine this region in detail. In order to distinguish two different APC polypeptides generated in an *in vitro* reaction, we constructed a tagged APC (HA-APC) containing an open reading frame coding for the HA epitope fused in frame to codon 1 to 1013 of APC (Fig. 3). This template and one of the templates coding for various fragments of APC were used for *in vitro* transcription-translation reactions. The translated products were immunoprecipitated with a monoclonal antibody against HA. Since only the HA-APC could be directly immunoprecipitated by the HA-specific monoclonal antibody, any other APC fragments present in the immunoprecipitates must be associated with HA-APC.

As shown in Fig. 4*A*, amino terminal fragments of APC were able to form complexes with the HA-APC when they were translated together. The *MCC* gene product, which was also predicted to form coiled-coil structures (1, 21), did not associate with HA-APC, demonstrating the specificity of this interaction. Moreover, an APC fragment containing codons 680 to 1110 did not associate with HA-APC, indicating that the amino terminus of APC was critical for the interaction (data not shown). Consistent with the result with cell lysates

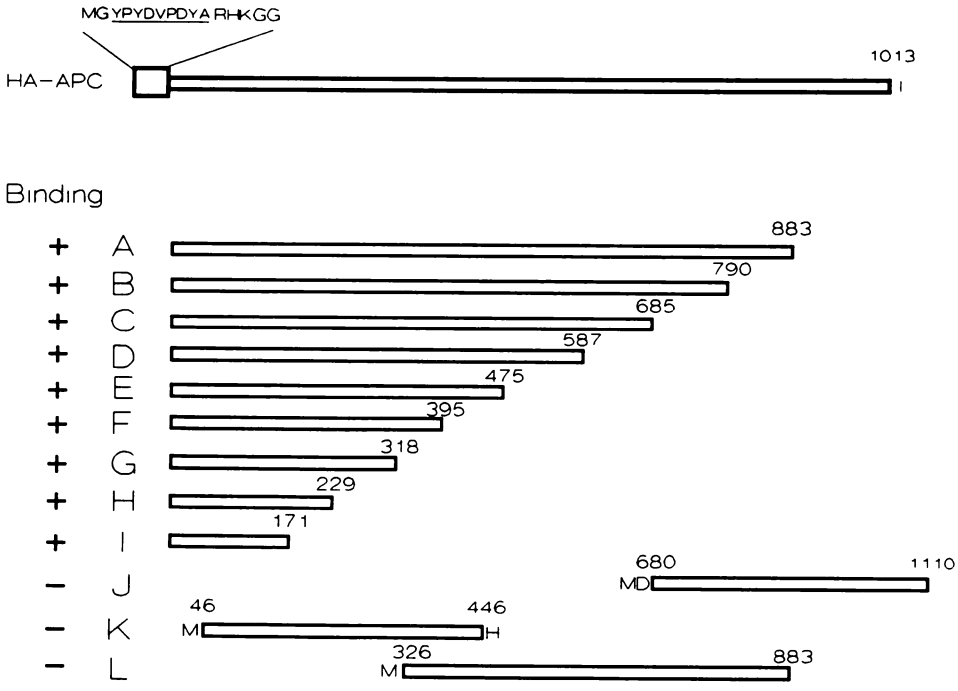

Fig. 3. APC proteins expressed *in vitro*. All the proteins start from the first codon of APC unless otherwise noted. *Numbers*, codons of APC. Any amino acids not encoded by APC are shown. The epitope for the anti-HA antibody used is *underlined*. + or –, the ability of each APC fragment to bind HA-APC *in vitro*.

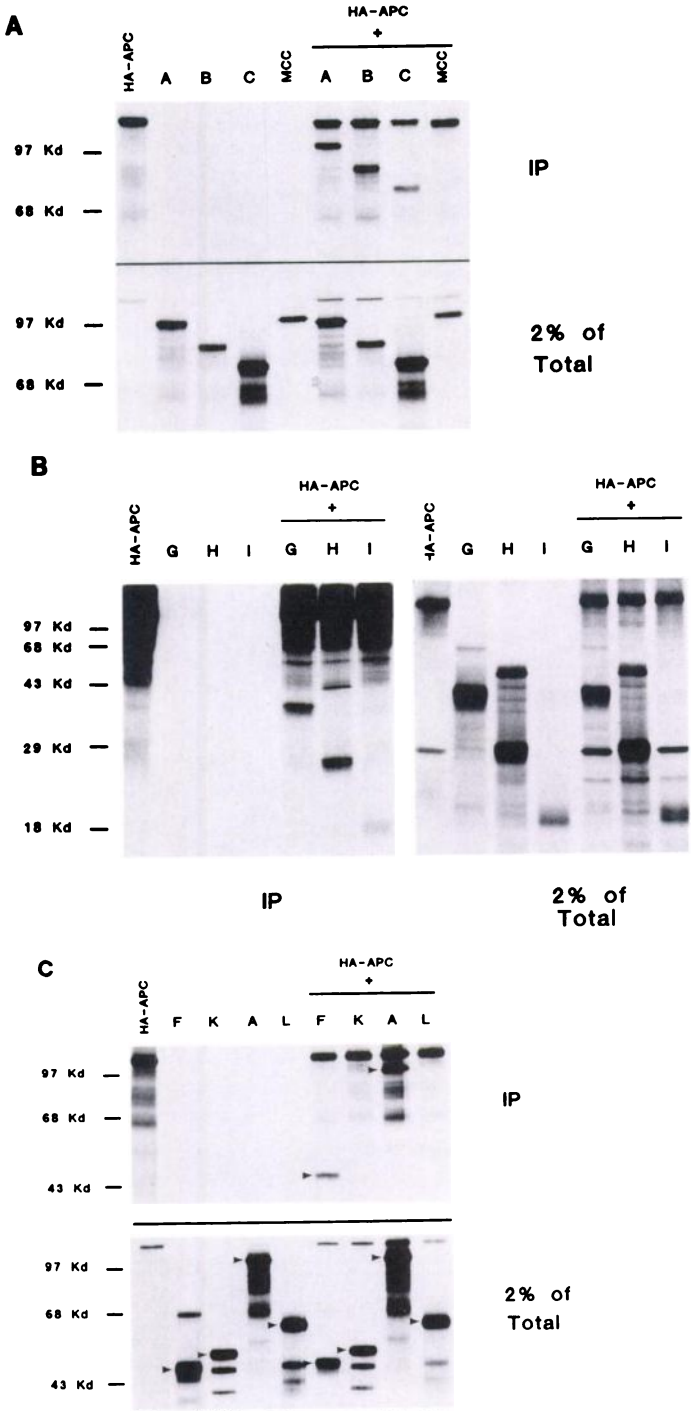

Fig. 4. Association of APC proteins expressed *in vitro*. *IP*, proteins present in immunoprecipitates using the HA antibody. *2% of total*, 2% of total *in vitro* translated proteins used for each immunoprecipitation. APC proteins translated *in vitro* (designated A, B, C, F, G, H, I, K, L) are illustrated in Fig. 3, and the template coding for the full length MCC protein has been described (22). *A*, association of truncated APC with HA-APC; *B*, the amino terminal 171 residues of APC is sufficient for association with HA-APC. *C*, amino terminal 45 residues of APC is required for association with HA-APC; *B* and *C* were intentionally overexposed to demonstrate the association between HA-APC and small APC fragments. *Arrowheads*, correct APC fragments.

described above, APC fragments did not associate with HA-APC when they were translated separately and then mixed together (data not shown).

The series of *in vitro* assays shown in Fig. 4*B* demonstrated that the first 171 residues of APC were sufficient for complex formation. Within this 171-residue region, there were two regions theoretically capable of forming coiled-coil structures, corresponding to two heptad-repeats (1, 3). To test whether the first heptad-repeat [codons 6 to 57 (3)] was required for the oligomerization of APC, a template coding for codons 46 to 446 of APC was generated and used in the assay. This amino terminally deleted APC could not associate with HA-APC (Fig. 4*C*). We also tested whether other coiled-coil regions of APC were able to mediate the protein-protein interaction. A template encoding amino acids 326 to 883 of APC, which included several coiled-coil regions corresponding to four heptad-repeats, was used in the assay. The product from this template could not associate with HA-APC (Fig. 4*C*).

These results indicate that truncated APC associates with wild type APC *in vivo* and suggest that the wild type APC gene product exists as an oligomer. The APC complex could also be demonstrated *in vitro* when polypeptides were cotranslated, suggesting a direct association between APC proteins. Fig. 3 summarizes the *in vitro* complex formation results. It was found that the first 171 residues of APC were sufficient (Fig. 4*B*) and that the first 45 residues were required (Fig. 4*C*) for the APC protein association. Almost all characterized germline mutations of the *APC* gene are nonsense mutations or small deletions or insertions which would produce truncations downstream of codon 171. Indeed, only 3 of 172 reported truncating germline mutations occur prior to codon 171 (5–11), and gross deletions and potential missense mutations are rare. Our results strongly suggest that, by association with the wild type APC, most mutant APC proteins have the potential to exert dominant negative effects upon the wild type *APC* gene product. However, it is possible that not all the mutant proteins have identical activities in this regard, perhaps because of the instability of some truncated APC proteins *in vivo* (19). In addition, very small truncated proteins resulting from mutations 5′ to codon 171 may not be able to associate with wild type APC. Patients carrying such mutations may have phenotypes different from that of typical FAP patients; *e.g.*, they might develop very few polyps. Families with an inherited disease similar to FAP, but with fewer than the usual number of polyps, have been reported, and this phenotype has been described as "variant" or "attenuated" forms of FAP (23, 24). It is noteworthy that Groden *et al.* (9) mentioned that one such case had a mutation at the 5′ end of APC, although the exact position has not yet been reported.

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