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APC Binds to the Novel Protein EB1

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Abstract

Mutations of the APC gene play a critical role in both sporadic and familial forms of colorectal cancer. The vast majority of these mutations result in the loss of the carboxy terminus of the protein. To further elucidate the function of APC, we searched for cellular proteins that associate with its carboxy terminus. One million human cDNA clones were screened with the use of the interaction trap two-hybrid system, and 67 clones were found to have a phenotype suggestive of an APC-interacting protein. Nucleotide sequence analysis revealed that 48 of these clones were derived from a single novel gene named EB1. The association of APC and EB1 proteins was confirmed with in vitro binding assays. mAbs against EB1 were then produced and used to demonstrate the association of APC and EB1 in vivo. The EB1 gene was predicted to encode a 268-amino acid protein without significant homology to proteins with known function. However, searches of nucleotide databases did identify evidence for at least two related human genes and a yeast homologue. This conservation suggests an essential function for EB1 that might provide clues to the mechanism through which APC suppresses colonic neoplasia.

Introduction

The APC gene was isolated by virtue of its alteration in familial and sporadic forms of colorectal cancer (1–4). Germline mutations of APC have been found in most cases of familial adenomatous polyposis, an autosomal dominantly inherited disease that predisposes patients to multiple colorectal polyps and cancer (reviewed in Ref. 5). Although patients with germline mutations of APC account for <1% of colorectal cancers in the United States, somatic mutations of APC occur in the great majority of sporadic colorectal tumors (6–9). These alterations appear to occur early in colorectal tumorigenesis because they can be identified in very small benign tumors and microscopic dysplastic foci (6, 10, 11). Virtually all germline and somatic APC alterations are predicted to result in the truncation of the APC protein due to either nonsense or frameshifting mutations (5–9). Likewise, mice carrying germline truncating mutations of APC are predisposed to intestinal tumors (12–14). Altogether, these results strongly suggest that APC mutations are an early and, perhaps, initiating event in the development of both sporadic and inherited forms of colorectal cancer.

Although disruption of normal APC function clearly plays an important role in colorectal tumorigenesis, what this function might be remains unclear. The APC gene is predicted to encode a protein of 2843 amino acids with limited functional homology to known proteins. The APC protein contains several Armadillo repeats that are shared by proteins with apparently diverse functions (3, 15). APC also contains several regions of heptad repeats, which could mediate protein oligomerization via coiled-coil structures (3). Indeed, the amino terminus of APC, which has a very strong potential for forming coiled-coil structures, has been shown to mediate the homooligomerization of APC (16, 17). APC has been shown recently to interact with catenins, cytoplasmic proteins that are essential for the cell adhesion activity of cadherin (18, 19). In addition, wild-type but not mutant forms of APC have been shown to associate with the microtubule cytoskeleton (20, 21).

Although the aforementioned biochemical characteristics of APC provide important clues to its function, other functions remain undefined. Because mutant APC proteins almost uniformly lack their carboxy terminus, it is likely that the carboxy terminus of APC interacts with proteins that are essential for its normal role. We describe here the use of the interaction trap two-hybrid system (22, 23) to identify a novel and highly conserved protein that associates with the carboxy terminus of APC.

Materials and Methods

Interactor Hunt. The interaction trap two-hybrid system, the cDNA library, and screening have been described (23). The bait was made by inserting a 2.5-kb EcoRI fragment of APC containing nucleotides 6498–8950 into the Smal site of LexA(l-202)+PL (24) after both ends were filled-in with the use of the Klenow fragment of DNA polymerase I.

GST Fusion Proteins. The pGST-EB1 expression vector was constructed with the use of an EcoRI fragment (nucleotides 317–899) of an EB1 cDNA clone isolated by the yeast two-hybrid system. After subcloning into the EcoRI site of pBluescript SK II, this fragment was excised as a BamHI-Sall fragment and inserted into the BamHI and Xhol sites of pGStag (25). The pGST-EB1B expression vector was constructed by inserting a 1.8-kb Sall-HindIII fragment (nucleotides 40–2091) of an EB1 cDNA clone isolated from human fetal brain cDNA library into the Sall and HindIII sites of pGStag. The pGST-APC expression vector was constructed by inserting the 2.5-kb EcoRI fragment of APC cDNA, identical to that used for making the bait for the two-hybrid screening, into the EcoRI site of pGStag. The pGStag-APC(X) vector was constructed by inserting the most 3' Xhol-EcoRI fragment of APC (nucleotides 7677–8950) as a Xhol-HindIII fragment isolated from an intermediate plasmid into the Sall and HindIII sites of pGStag. The expression and purification of fusion proteins were performed as described (19).

PCR and in Vivo Expression of EB1. The coding region of EB1 was amplified with the use of the upstream primer 5'-GGATCCTAATACGACTCACTATAGGGAGACCCACACAGGCGAAGCTATCTACTC-3' and the downstream primer 5'-ATTTTTCCACTAGGTCGCGG-3'. The upstream primer contained the promoter for the T7 RNA polymerase and the first 20 nucleotides of the EB1 coding sequence. The downstream primer was located at the 3' untranslated region of EB1. The PCR reaction was carried out with the use of a cDNA clone template for 35 cycles of 30 s at 95°C, 1 min at 50°C,
and 1 min at 70°C. The PCR product was used directly in a coupled in vitro transcription and translation reaction as described (26).

**In Vitro Binding Assay.** Cell extracts prepared from metabolically labeled human colorectal cancer cell lines SW480 and HCT116 were used for the in vitro binding assay. Metabolic labeling, preparation of cell lysates, in vitro binding, and peptide mapping were performed as described (19).

mAbs. The three EBI mAbs AE9, EA3, and GD10 were derived from mice immunized with the GST-EB1 fusion protein. Immunization of mice, cell fusion, and the preparation of mAbs were performed as described (27). All three mAbs were found to specifically recognize EBI by both immunoblot and immunoprecipitation analyses. The anti-APC mAb FE9 (APC Ab-1; Oncogene Science, Inc.) has been described previously (27).

**In Vivo Binding Assay.** SW480 cells were transiently transfected with pcMV-APC (20) or pcMV-EB1. The pcMV-EB1 vector was derived by cloning a PCR product containing EBI nucleotides 62–871 into the BamHI site of pcMV-NEO-BAM. PCR was performed with the following primers that were engineered to be included under the unlimited 8gIII sites: 5'-CGAGATCTGAAAGCTTGTAGATGGGTAAT-3' and 5'-CGAGATCTTATATCCTTCTTGTAGATGGGTAAT-3'. To eliminate the possibility of PCR errors, the sequence of pCMV-NEO-BAM. PCR was performed with the following primers that were used for FISH. Hybridization was carried out with the use of a modified procedure of Pinkel et al. (28) as described previously (29).

**Database Searches and Alignments.** The NCBI's nonredundant nucleotide, nonredundant protein, and DBEST databases (1/19/95 releases) were searched with the use of the BLASTN, BLASTP, and TBLASTN basic local alignment search software, respectively (30). Multiple alignments were performed with the use of the MACAW multiple alignment construction and analysis software, version 2.03 (31).

**Results and Discussion.** We used the interaction trap, a yeast two-hybrid system (22, 23), to select human proteins encoded by a HeLa cDNA library that interact with the carboxyl terminus (codons 2167–2843) of APC. A total of 90 clones with the appropriate phenotype were identified after screening 1 million transformants. The cDNAs isolated from 67 of these 90 clones were able to confer the correct phenotype after being transreconstituted in a yeast two-hybrid system (22, 23). To determine if this binding was specific and not a consequence of the interaction trap, we transiently transfected the neutrophil cell line 32D with EBI cDNA cloned into pCMV-APC (20) or pCMV-EB1. The pCMV-EB1 vector was derived by cloning a PCR product containing EBI nucleotides 62–871 into the BamHI site of pcMV-NEO-BAM. The pCMV-APC vector was derived by cloning a PCR product containing APC nucleotides 1–1677 into the BamHI site of pcMV-NEO-BAM. PCR was performed with the following primers that were used for FISH. Hybridization was carried out with the use of a modified procedure of Pinkel et al. (28) as described previously (29).

**Chromosomal Localization of EBI by FISH.** Three EBI genomic clones (EBI–922, EBI–923, and EBI–924) were obtained by PCR screening of a P1 library (Genome Systems, Inc.) with the use of primers (5'-AAACCAAGAAGTGGTTATACGAC-3' and 5'-ATTTCTCCTACGATTGTCC-3') designed to amplify EBI nucleotides 1102–1205. Total EBI–923 DNA was labeled with Biotin-16-DUTP by nick translation and used for FISH. Hybridization was carried out with the use of a modified procedure of Pinkel et al. (28) as described previously (29).

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Fig. 2. In vitro binding of EB1 to APC. A, binding of cellular APC to GST-EB1 fusion protein. SW480 and HCT116 are human colorectal cancer cell lines that express truncated and full-length APC, respectively (19). Protein from total cell lysates (–) or protein bound by GST-EB1 fusion protein (GST-EB1) was analyzed by Western blot analysis with the APC-specific mAb FE9 (19). B, binding of EB1 to GST-APC fusion protein. GST-CTN has been described (19) and was used as a negative control. SW480 and HCT116 cellswere metabolically labeled with [15S]Met and incubated with the GST fusion proteins as indicated. In vitro transcribed and translated EB1 (in vitro) was run on gel directly (–) or following binding to GST-APC(X) fusion protein as indicated. Proteins were detected by fluorography. C, one-dimensional peptide mapping. Cellular (SW480 and HCT116) and in vitro-translated (in vitro) EB1 proteins were isolated by binding to GST-APC(X) and subjected to one-dimensional peptide mapping as described (19). kD, molecular weight in kilodaltons.

Fig. 3. In vivo association of APC and EB1. SW480 cells were transiently transfected with expression vectors for EB1 or APC as indicated. The parental expression vector pCMV-NEO-BAM (pCMV) was used to equalize the total amount of DNA transfected. Lysates prepared from these transfected cells were used directly (total) or after immunoprecipitation with a mAb against hemagglutinin (HA) as a negative control or an EB1-specific mAb (EB1). Detection of APC was carried out by immunoblotting with the use of the APC-specific mAb FE9. MT and FL, truncated and full-length APC, respectively.

Fig. 4. FISH localizing EB1 to chromosome 20q11.2. Left panel, an ideogram of a G-banded human chromosome 20 with the band q11.2 bracketed. Top right panel, fluorescent signals localizing EB1 to chromosome 20. Bottom right panel, a G-banded human chromosome 20 localizing EB1 to 20q11.2.
formed into the test strain of yeast. The nucleotide sequences of both ends of each cDNA were determined and compared to each other. Forty-eight of these cDNAs were found to be derived from a single gene and could be separated into 11 groups according to their 5' ends (Fig. 1). As expected, the fusion proteins encoded by two independent cDNA clones representative of this gene did not interact with APC (Fig. 1). As expected, this fusion protein was able to associate with the truncated APC protein found in SW480 cells (Fig. 2A). This result clearly showed that EBl interacts with cellular APC and that this interaction required the carboxyl terminus of APC. To confirm and extend these two-hybrid results, we tested the interaction between EBl and APC using an in vitro binding assay. The carboxyl terminal 163 residues of EBl was expressed as a GST fusion protein (GST-EBl) in Escherichia coli. This fragment of EBl was expected to bind APC because it included more of EBl than several carboxyl terminal 284 residues of APC were sufficient for EBl binding and provided additional evidence that the first methionine codon in the EBl cDNA was the translation initiation site.

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To further characterize the association between APC and EB1, three mAbs against EB1 (AE9, EA3, and GD10) were generated. When used for Western blot analysis, all three of these antibodies detected a Mr 30,000 protein in total cell lysates, which associated with GST-APC but not with a control protein, GST-CTN (data not shown). EB1 protein was detected in several human colon cancer cell lines as well as in the human kidney fibroblast cell line 293, the canine kidney epithelial cell line MDCK, and the mouse fibroblast cell line NIH3T3 (data not shown).

To demonstrate an in vivo interaction between EB1 and APC in mammalian cells, SW480 cells were transiently transfected with vectors expressing APC or EB1. The association between these two proteins was examined by immunoprecipitation with the use of the EB1-specific antibody EA3, followed by immunoblotting with the APC-specific antibody FE9. The coimmunoprecipitation of APC and EB1 was clearly demonstrated when cells were transfected with both expression vectors but not when either one was omitted (Fig. 3).

The chromosomal localization of EB1 was determined by FISH. Three P1 clones for EB1 were isolated from a P1 human genomic library by PCR, and one was used as the probe for FISH analysis. Sixteen of a total of 17 metaphase cells examined displayed double fluorescent signals (i.e., one on each chromatid) on the proximal long arm of chromosome 20. The same cells used for FISH had been previously G-banded and photographed. Comparison of these results demonstrated that EB1 resided at 20q11.2 (Fig. 4).

Searches of nucleotide databases indicated that EB1 had not been characterized previously. However, there were several ESTs that were almost identical to parts of the 3'-untranslated region of EB1. Interestingly, there were also five ESTs that were similar but not identical to the coding region of EB1. These ESTs likely represented novel EB1-related genes rather than sequencing mistakes because there were numerous nucleotide substitutions that preserved the encoded amino acids of EB1 in these ESTs. These five ESTs could be divided into three contigs that represented at least two different EB1-related genes (Fig. 5A). Searches of NCBI's nonredundant protein database with EB1 identified three proteins with statistically significant (P < 0.05) multiple regions of homology. These were a calcium channel protein from carp [Protein Identification Resource (PIR) accession no. A37860; P = 0.0075], a bacterial RNA polymerase α chain homologue (PIR accession no. JN0445; P = 0.0028), and YerOlop from carp [Protein Identification Resource (PIR) accession no. A37860; P = 0.0075], a bacterial RNA polymerase α chain homologue (PIR accession no. JN0445; P = 0.0028), and YerOlop from carp.

To demonstrate the association of EB1 with pRB, we used an interaction trap two-hybrid system to identify a novel protein interaction (32, 33). However, it is clear that additional experiments are necessary to determine the physiological significance of the EB1/APC interaction.

Because almost all APC mutations result in truncation of the APC protein, the vast majority of APC mutants would be expected to lose the ability to interact with EB1. This observation suggests that the interaction between APC and EB1 may be important for the normal tumor suppressor function of APC and raises the possibility that mutations of EB1 could also result in tumorigenesis. Additional characterization of EB1 and the interaction between APC and EB1 may provide additional insights into colorectal tumorigenesis.

References


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