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APC Binds to the Novel Protein EB1[1]

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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 carboxyl terminus of the protein. To further elucidate the function of APC, we searched for cellular proteins that associate with its carboxyl 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*[6] 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 carboxyl terminus, it is likely that the carboxyl 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 carboxyl 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 *Eco*RI fragment of *APC* containing nucleotides 6498–8950 into the *Sma*I site of LexA(1–202)+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 *Eco*RI fragment (nucleotides 317–899) of an *EB1* cDNA clone isolated by the yeast two-hybrid system. After subcloning into the *Eco*RI site of pBluescript SK II, this fragment was excised as a *Bam*HI-*Sal*I fragment and inserted into the *Bam*HI and *Xho*I sites of pGSTag (25). The pGST-EB1B expression vector was constructed by inserting a 1.8-kb *Sal*I-*Hin*dIII fragment (nucleotides 40–2091) of an *EB1* cDNA clone isolated from human fetal brain cDNA library into the *Sal*I and *Hin*dIII sites of pGSTag. The pGST-APCE expression vector was constructed by inserting the 2.5-kb *Eco*RI fragment of *APC* cDNA, identical to that used for making the bait for the two-hybrid screening, into the *Eco*RI site of pGSTag. The pGST-APC(X) vector was constructed by inserting the most 3′ *Xho*I-*Eco*RI fragment of APC (nucleotides 7677–8950) as a *Xho*I-*Hin*dIII fragment isolated from an intermediate plasmid into the *Sal*I and *Hin*dIII sites of the pGSTag. The expression and purification of fusion proteins were performed as described (19).

PCR and *in Vitro* Expression of *EB1*. The coding region of *EB1* was amplified with the use of the upstream primer 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGCAGTGAACGTATACTC-3′ and the downstream primer 5′-ATTTCTCCACTGAGGTCGC-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,

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[6] The abbreviations used are: APC, adenomatous polyposis coli; EST, expressed sequence tag; GST, glutathione *S*-transferase; FISH, fluorescence *in situ* hybridization; NCBI, National Center for Biotechnology Information.

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 EB1 mAbs AE9, EA3, and GD10 were derived from mice immunized with the GST-EB1B 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 EB1 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 *EB1* nucleotides 62–871 into the *Bam*HI site of pCMV-NEO-BAM. PCR was performed with the following primers that were engineered to include the underlined *Bgl*II sites: 5′-CGAGATCTAA-GATGGCAGTGAACGTATAC-3′ and 5′-GCAGATCTTTAATACTCT-TCTTGATCCTCC-3′. To eliminate the possibility of PCR errors, the sequence of the *EB1* fragment cloned into pCMV-EB1 was verified by nucleotide sequencing. Transient tranfections, preparation of cell lysates, immunoprecipitation, and Western blot analysis were performed as described (16, 19, 20).

Chromosomal Localization of EB1 by FISH. Three *EB1* genomic clones (EB1–922, EB1–923, and EB1–924) were obtained by PCR screening of a P1 library (Genome Systems, Inc.) with the use of primers (5′-AAAACA-GAGAGGCTGACCG-3′ and 5′-ATTTCTCCACTGAGGTCGC-3′) designed to amplify *EB1* nucleotides 1102–1205. Total EB1–923 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).

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 retrans-


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   1                                                                           Met Ala Val Asn Val Tyr     6
   1   A CGA GAC GAA GAC GGA ACC GGA GCC GGT TGC GGG CAG TGG ACG CGG TTC TGC CGA GAG CCG AAG ATG GCA GTG AAC GTA TAC    82

   7 Ser Thr Ser Val Thr Ser Asp Asn Leu Ser Arg His Asp Met Leu Ala Trp Ile Asn Glu Ser Leu Gln Leu Asn Leu Thr Lys    34
  83 TCA ACG TCA GTG ACC AGT GAT AAC CTA AGT CGA CAT GAC ATG CTG GCC TGG ATC AAT GAG TCT CTG CAG TTG AAT CTG ACA AAG   166

  35 Ile Glu Gln Leu Cys Ser Gly Ala Ala Tyr Cys Gln Phe Met Asp Met Leu Phe Pro Gly Ser Ile Ala Leu Lys Lys Val Lys    62
 167 ATC GAA CAG TTG TGC TCA GGG GCT GCG TAT TGT CAG TTT ATG GAC ATG CTG TTC CCT GGC TCC ATT GCC TTG AAG AAA GTG AAA   250

  63 Phe Gln Ala Lys Leu Glu His Glu Tyr Ile Gln Asn Phe Lys Ile Leu Gln Ala Gly Phe Lys Arg Met Gly Val Asp Lys Ile    90
 251 TTC CAA GCT AAG CTA GAA CAC GAG TAC ATC CAG AAC TTC AAA ATA CTA CAA GCA GGT TTT AAG AGA ATG GGT GTT GAC AAA ATA   334

  91 Ile Pro Val Asp Lys Leu Val Lys Gly Lys Phe Gln Asp Asn Phe Glu Phe Val Gln Trp Phe Lys Lys Phe Phe Asp Ala Asn   118
 335 ATT CCT GTG GAC AAA TTA GTA AAA GGA AAG TTT CAG GAC AAT TTT GAA TTC GTT CAG TGG TTC AAG AAG TTT TTC GAT GCA AAC   418

 119 Tyr Asp Gly Lys Asp Tyr Asp Pro Val Ala Ala Arg Gln Gly Gln Glu Thr Ala Val Ala Pro Ser Leu Val Ala Pro Ala Leu   146
 419 TAT GAT GGA AAA GAC TAT GAC CCT GTG GCT GCC AGA CAA GGT CAA GAA ACT GCA GTG GCT CCT TCC CTT GTT GCT CCA GCT CTG   502

 147 Asn Lys Pro Lys Lys Pro Leu Thr Ser Ser Ser Ala Ala Pro Gln Arg Pro Ile Ser Thr Gln Arg Thr Ala Ala Ala Pro Lys   174
 503 AAT AAA CCG AAG AAA CCT CTC ACT TCT AGC AGT GCA GCT CCC CAG AGG CCC ATC TCA ACA CAG AGA ACC GCT GCG GCT CCT AAG   586

 175 Ala Gly Pro Gly Val Val Arg Lys Asn Pro Gly Val Gly Asn Gly Asp Asp Glu Ala Ala Glu Leu Met Gln Gln Val Asn Val   202
 587 GCT GGC CCT GGT GTG GTG CGA AAG AAC CCT GGT GTG GGC AAC GGA GAC GAC GAG GCA GCT GAG TTG ATG CAG CAG GTC AAC GTA   670

 203 Leu Lys Leu Thr Val Glu Asp Leu Glu Lys Glu Arg Asp Phe Tyr Phe Gly Lys Leu Arg Asn Ile Glu Leu Ile Cys Gln Glu   230
 671 TTG AAA CTT ACT GTT GAA GAC TTG GAG AAA GAG AGG GAT TTC TAC TTC GGA AAG CTA CGG AAC ATT GAA TTG ATT TGC CAG GAG   754

 231 Asn Glu Gly Glu Asn Asp Pro Val Leu Gln Arg Ile Val Asp Ile Leu Tyr Ala Thr Asp Glu Gly Phe Val Ile Pro Asp Glu   258
 755 AAC GAG GGG GAA AAC GAC CCT GTA TTG CAG AGG ATT GTA GAC ATT CTG TAT GCC ACA GAT GAA GGC TTT GTG ATA CCT GAT GAA   838

 259 Gly Gly Pro Gln Glu Glu Gln Glu Glu Tyr                                                                          268
 839 GGG GGC CCA CAG GAG GAG CAA GAA GAG TAT TAA CAG CCT GGA CCA GCA GAG CAA CAT CGG AAT TCT TCA CTC CAA ATC ATG TGC   922
 923 TTA ACT GTA AAA TAC TCC CTT TTG TTA TCC TTA GAG GAC TCA CTG GTT TCT TTT CAT AAG CAA AAA GTA CCT CTT CTT AAA GTG  1006
1007 CAC TTT GCA GAC GTT TCA CTC CTT TTC CAA TAA GTT TGA GTT AGG AGC TTT TAC CTT GTA GCA GAG CAG TAT TAA CAT CTA GTT  1090
1091 GGT TCA CCT GGA AAA CAG AGA GGC TGA CCG TGG GGC TCA CCA TGC GGA TGC GGG TCA CAC TGA ATG CTG GAG AGA TGT ATG TAA  1174
1175 TAT GCT GAG GTG GCG ACC TCA GTG GAG AAA TGT AAA GAC TGA ATT GAA TTT TAA GCT AAT GTG AAA TCA GAG AAT GTT GTA ATA  1258
1259 AGT AAA TGC CTT AAG AGT ATT TAA AAT ATG CTT CCA CAT TTC AAA ATA TAA AAT GTA ACA TGA CAA GAG ATT TTG CGT TTG ACA  1342
1343 TTG TGT CTG GGA AGG AAG GGC CAG ACC TTG GAA CCT TTG GAA CCT GCT GTC AAC AGG TCT TAC AGG GCT GCT TGA ACC CTC ATA  1426
1427 GGC CTA GGC TTT GGT CTA AAA GGA ACA TTT AAA AAG TTG CCC TGT AAA GTT ATT TGG TGT CAT TGA CCA ATT GCA TCC CAG CTA  1510
1511 AAA AGC AAG AGG CAT CGT TGC CTG GAT AAT AGA GGA TGT GTT TCA GCC CTG AGA TGT TAC AGT TGA AGA GCT TGG TTT CAT TGA  1594
1595 GCA TTT CTC TAT TTT TCC AGT TAT CCC GAA ATT TCT ATG TAT TAT TTT TTG GGG AAG TGA GGT GTG CCC AGT TTT TTA ATC TAA  1678
1679 CAA CTA CTT TTG GGG ACT TGC CCA CAT CTC TGG GAT TTG AAT GGG GAT TGT ATC CCA TTT TAC TGT CTT TTA GGT TTA CAT TTA  1762
1763 CCA CGT TTC TCT TCT CTG CTC CCC TTG CCC ACT GGG ACT CCT CTT TGG CTC CTT GAA GTT TGC TGC TTA GAG TTG GAA GTG CAG  1846
1847 CAG GCA GGT GAT CAT GCT GCA AGT TCT TTC TGG ACC TCT GGC AAA GGG AGT GGT CAG TGA AGG CCA TCG TTA CCT TGG GAT CTG  1930
1931 CCA GGC TGG GGT GTT TTC GGT ATC TGC TGT TCA CAG CTC TCC ACT GTA ATC CGA ATA CTT TGC CAG TGC ACT AAT CTC TTT GGA  2014
2015 GAT AAA ATT CAT TAG TGT GTT ACT AAA TGT TAA TTT TCT TTT GCG GAA AAT ACA GTA CCG TGT CTG AAT TAA TTA TTA ATA TTT  2098
2099 AAA ATA CTT CAT TCC TTA ACT CTC CCT CAT TTG CTT TGC CCA CAG CCT ATT CAG TTC CTT TGT TTG GCA GGA TTC TGC AAA ATG  2182
2183 TGT CTC ACC CAC TAC TGA GAT TGT TCA GCC CCT GAT GTA TTT GTA TTG ATT TGT TTC TGG TGG TAG CTT GTC CTG AAA TGT GTG  2266
2267 TAG AAA GCA AGT ATT TTA TGA TAA AAA TGT TGT GTA GTG CAT GCT CTG TGT GGA ATT CAG AGG AAA ACC CAG ATT CAG TGA TTA  2350
2351 ACA ATG CCA AAA AAT GCA AGT AAC TAG CCA TTG TTC AAA TGA CAG TGG TGC TAT TTC TCT TTT GTG GCC TTT TAG ACT TTT GTT  2434
2435 GCC CTA AAA TTC CAT TTT ATT GGG AAC CCA TTT TCC ACC TGG TCT TTC TTG ACA GGG TTT TTT TCT ACT TTA AAC AGT TTC TAA  2518
2519 ATA AAA TTC TGT ATT TCA AAA A                                                                                    2540
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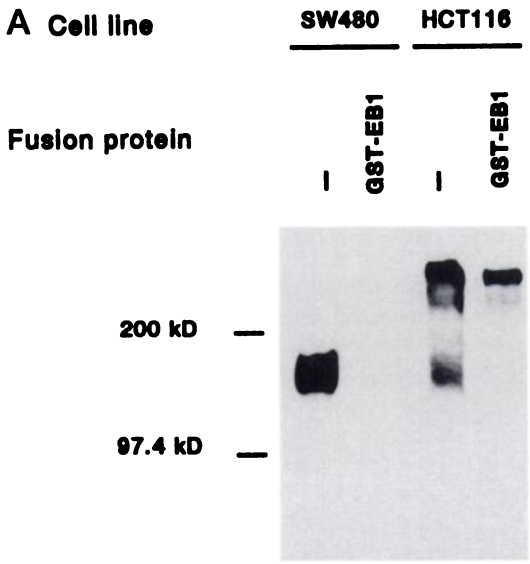
Fig. 1. Nucleotide and predicted amino acid sequence of *EB1*. *Arrowheads* above the sequences, 5′ termini of different *EB1* cDNA clones isolated by yeast two-hybrid screening. The predicted amino acid sequence begins at nucleotide 65 and ends at nucleotide 868. The nucleotide sequence has been deposited with Genbank (accession no. U24166).



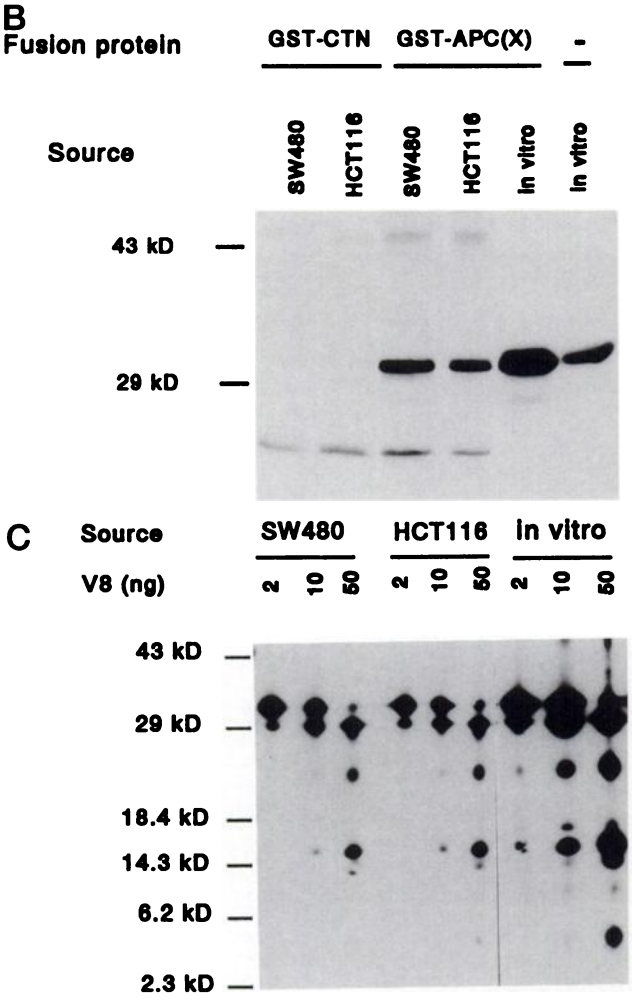


Fig. 2. *In vitro* binding of EB1 to APC. *A*, binding of cellular APC to GST-EB1 fusion protein. SW480 and HCT116 are human colorectal cancer cell lines that express truncated and full-length APC, respectively (19). Protein from total cell lysates (−) or protein bound by GST-EB1 fusion protein (GST-EB1) 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 cells were metabolically labeled with [^{35}S]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.

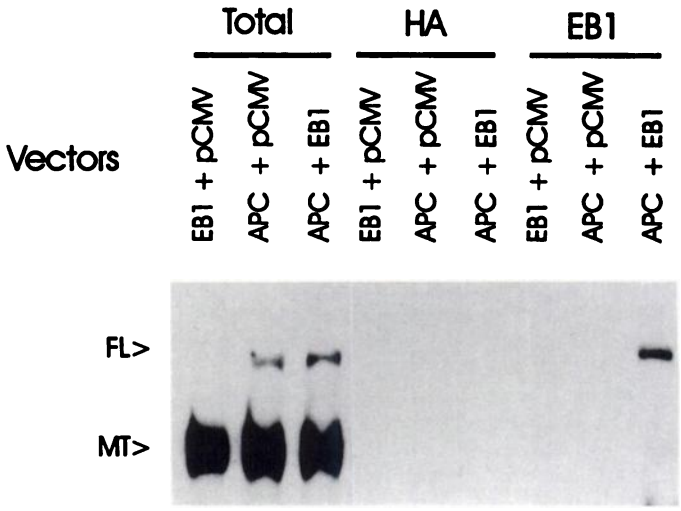


Fig. 3. *In vivo* association of APC and EB1. SW480 cells were transiently transfected with expression vectors for EB1 or APC as indicated. The parental expression vector pCMV-NEO-BAM (pCMV) was used to equalize the total amount of DNA transfected. Lysates prepared from these transfected cells were used 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.

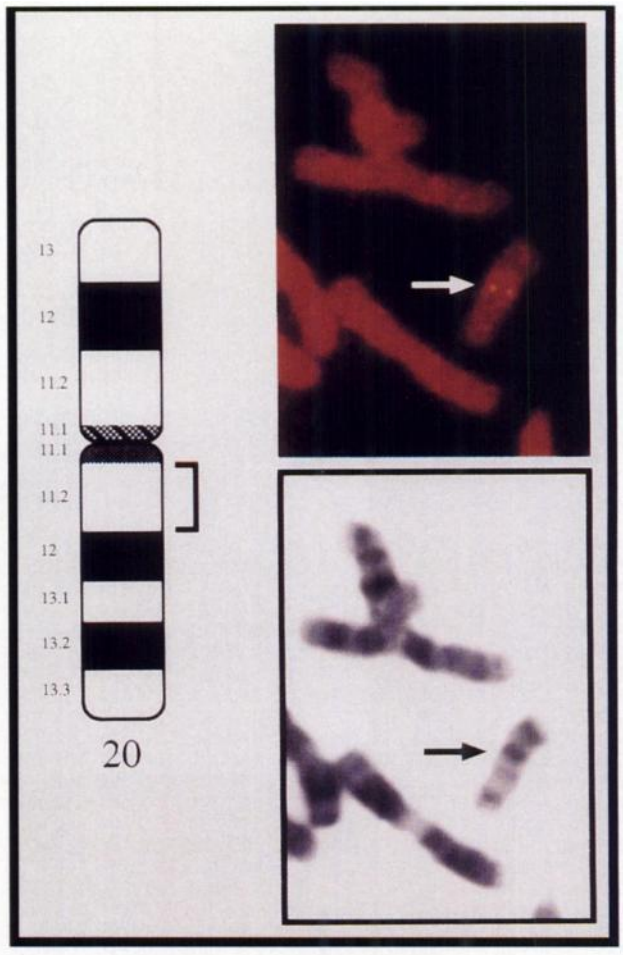


Fig. 4. FISH localizing *EB1* to chromosome 20q11.2. *Left panel*, an ideogram of a G-banded human chromosome 20 with the band q11.2 bracketed. *Top right panel*, fluorescent signals localizing *EB1* to chromosome 20. *Bottom right panel*, a G-banded human chromosome 20 localizing *EB1* to 20q11.2.

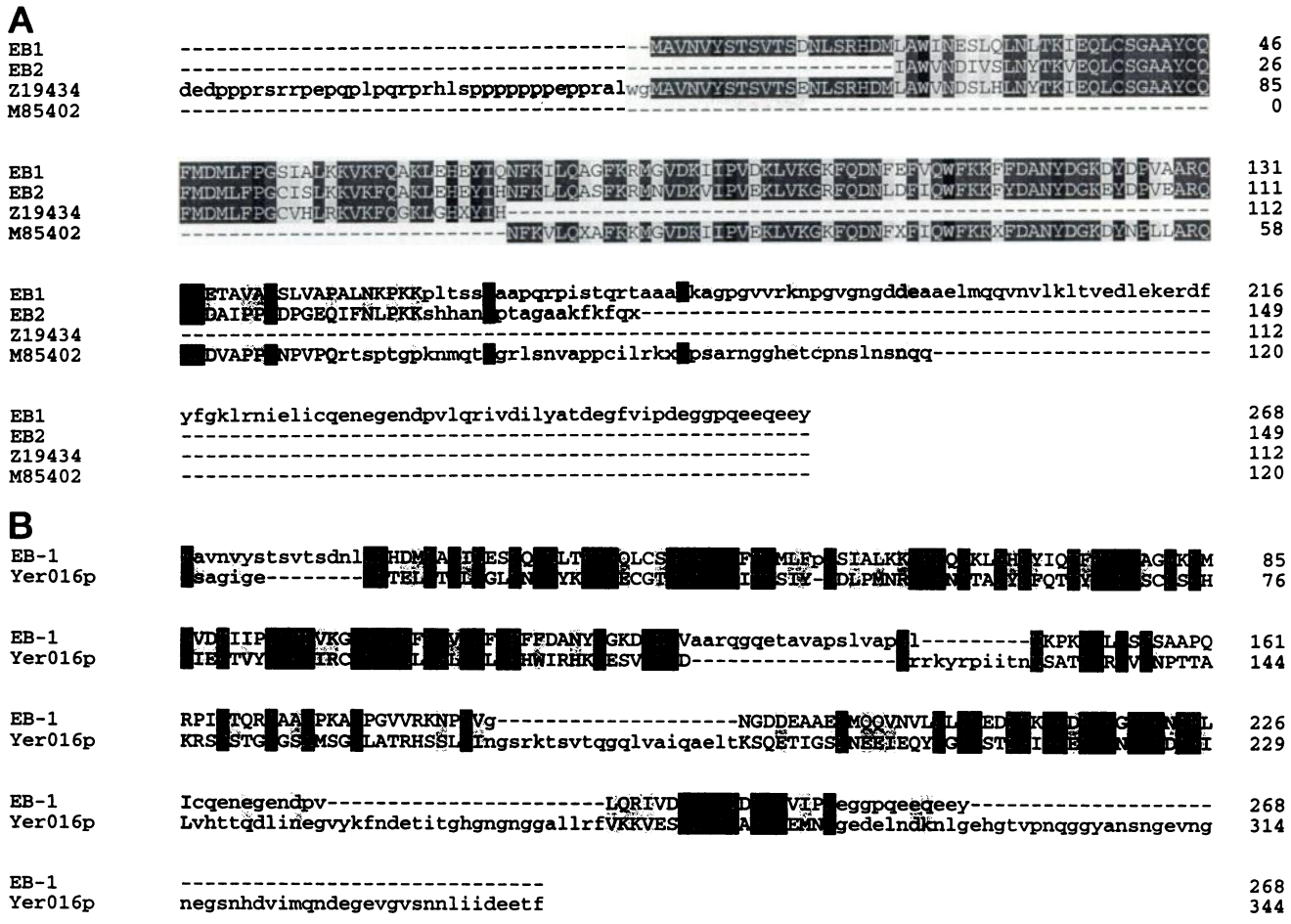


Fig. 5. Amino acid sequences homology among EB1 homologues. *A*, amino acid sequence comparison among human EB1 homologues. *EB2*, the amino acid sequence predicted from the nucleotide sequence of a contig of three different ESTs (Z46175, T17004, and Z42534.) The Z19434 and M85402 lines show the predicted amino acid sequences of these two ESTs, respectively. Because of the lack of overlap between Z19434 and M85402, we could not determine whether they represented one or two genes. ---, no sequence information was available at that position. *B*, amino acid sequence comparison between human EB1 and a potential yeast EB1 homologue. The sequence of Yer016p is predicted from an open reading frame from yeast chromosome V as described in the text. ---, gap introduced to allow the best alignment between the two sequences. In both *A* and *B*, blocks of homology are capitalized and shaded according to their mean scores.

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 residues 6–1013 when tested with the use of the same two-hybrid assay. We chose to characterize this gene in detail and named it *EB1*.

Northern blot analysis with probes to *EB1* identified a single 2.4-kb transcript (data not shown). Because the largest *EB1* cDNA obtained by two-hybrid screening was 1.4 kb, we screened a human fetal brain cDNA library to isolate the full-length cDNA. None of the newly isolated cDNA clones had additional 5′ nucleotide sequences, but many of them had additional 3′ nucleotide sequences extending the length of the cloned message to 2.4 kb. Furthermore, no additional 5′ sequences were identified by 5′-rapid amplification of cDNA ends (RACE), with the use of three different sources of mRNA (data not shown). Together, these results suggested that we had isolated the full-length cDNA for *EB1*. Nucleotide sequence analysis of the overlapping cDNA clones revealed an open reading frame extending from nucleotide 1 to 868 (Fig. 1). If translation initiated at the first methionine, *EB1* would be predicted to encode a protein of 268 amino acids with a predicted molecular weight of 30,000.

To confirm and extend these two-hybrid results, we tested the interaction between EB1 and APC using an *in vitro* binding assay. The carboxyl terminal 163 residues of EB1 was expressed as a GST fusion protein (GST-EB1) in *Escherichia coli*. This fragment of EB1 was expected to bind APC because it included more of EB1 than several of the *EB1* cDNA clones originally isolated by the yeast interaction trap method. As expected, this fusion protein was able to associate with the full-length APC protein from HCT116 cell lysates but not with the truncated APC protein found in SW480 cells (Fig. 2*A*). This result clearly showed that EB1 interacts with cellular APC and that this interaction required the carboxyl terminus of APC.

To test whether APC could bind endogenous EB1, we expressed the carboxyl terminus of APC as GST fusion proteins [APC codons 2167–2843 as GST-APCE and APC codons 2560–2843 as GST-APC(X)] and used them in an *in vitro* binding assay. These APC fusion proteins were able to bind a M_r 30,000 cellular protein with identical mobility to the EB1 expressed *in vitro* (Fig. 2*B* and data not shown). To confirm that this M_r 30,000 protein was indeed EB1, we compared the one-dimensional peptide map of the M_r 30,000 protein with that of EB1 expressed *in vitro*. The peptide maps of these proteins were identical (Fig. 2*C*). This result indicated that the most carboxyl terminal 284 residues of APC were sufficient for EB1 binding and provided additional evidence that the first methionine codon in the *EB1* cDNA was the translation initiation site.

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 M_r 30,000 protein in total cell lysates, which associated with GST-APCE 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. 5*A*). 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 σ chain homologue (PIR accession no. JN0445; $P = 0.0028$), and Yer016p ($P = 2.4 \times 10^{-53}$). *Yer016p* is a putative gene identified in a 66,030-bp *Saccharomyces cerevisiae* chromosome V cosmid contig (Genbank accession no. U18778). The predicted Yer016p protein shared five blocks of similarity with EB1 and could represent a yeast homologue of *EB1* (Fig. 5*B*). Together, these data suggest that *EB1* is a member of a conserved multigene family.

We used the interaction trap two-hybrid system to identify a novel protein that interacts with the carboxyl terminus of APC. The interaction between APC and EB1 was confirmed by both binding cellular APC to recombinant EB1 and by binding cellular EB1 to recombinant APC (Fig. 2). The association between EB1 and APC in mammalian cells was also clearly demonstrated in cells cotransfected with vectors expressing these two proteins (Fig. 3). However, we have not been able to detect the association between endogenous full-length APC and EB1 by coimmunoprecipitation experiments. The reason for failure to detect the association between endogenous APC and EB1 may be technical. This is consistent with our inability to coimmunoprecipitate APC and EB1 from cell lysates prepared from yeast clones with clear functional evidence for an APC-EB1 interaction and may reflect the sensitivity of the interaction trap, which can detect protein-protein associations with dissociation constants on the order 10^{-6} (23). Similar reasons have been suggested for the failure to demonstrate association between pRB and RBP2 by coimmunoprecipitation (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

1. Groden, J., Thliveris, A., Samowitz, W., Calson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., Le Paslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. Identification and characterization of the familial adenomatous polyposis coli gene. Cell, *66:* 589–600, 1991.
2. Joslyn, G., Calson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., Le Paslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. Identification of deletion mutations and three new genes at the familial polyposis locus. Cell, *66:* 601–613, 1991.
3. Kinzler, K. W., Nilbert, M. C., Su, L-K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I., and Nakamura, Y. Identification of *FAP* locus genes from chromosome 5q21. Science (Washington DC), *253:* 661–665, 1991.
4. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P., Markham, A., Kurch, A. J., Petersen, G., Hamilton, S. R., Nilbert, M. C., Levy, D. B., Bryan, T. M., Preisinger, A. C., Smith, K. J., Su, L-K., Kinzler, K. W., and Vogelstein, B. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science (Washington DC), *253:* 665–669, 1991.
5. Nagase, H., and Nakamura, Y. Mutations of the *APC* (adenomatous polyposis coli) gene. Hum. Mutat., *2:* 425–434, 1993.
6. Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., and Kinzler, K. W. APC mutations occur early during colorectal tumorigenesis. Nature (Lond.), *359:* 235–237, 1992.
7. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. Hum. Mol. Genet., *1:* 229–233, 1992.
8. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., Mishima, Y., Mori, T., and Koike, M. Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. Cancer Res., *54:* 3011–3020, 1994.
9. De Benedetti, L., Sciallero, S., Gismondi, V., James, R., Bafico, A., Biticchi, R., Masetti, E., Bonelli, L., Heouaine, A., Picasso, M., Groden, J., Robertson, M., Risio, M., Caprilli, R., Bruzzi, P., White, R. L., Aste, H., Santi, L., Varesco, L., and Ferrara, G. B. Association of *APC* gene mutations and histological characteristics of colorectal adenomas. Cancer Res., *54:* 3553–3556, 1994.
10. Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Molecular determinants of dysplasia in colorectal lesions. Cancer Res., *54:* 5523–5526, 1994.
11. Smith, A. J., Stern, H. S., Penner, M., Hay, K., Mitri, A., Bapat, B. V., and Gallinger, S. Somatic *APC* and K-*ras* codon 12 mutations in aberrant crypt foci from human colons. Cancer Res., *54:* 5527–5530, 1994.
12. Moser, A. R., Pitot, H. C., and Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science (Washington DC), *247:* 322–324, 1990.
13. Su, L-K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. Science (Washington DC), *256:* 668–670, 1992.
14. Fodde, R., Edelmann, W., Yang, K., van Leeuwen, C., Carlson, C., Renault, B., Breukel, C., Alt, E., Lipkin, M., Meera Khan, P., and Kucherlapati, R. A targeted chain-termination mutation in the mouse *Apc* gene results in multiple intestinal tumors. Proc. Natl. Acad. Sci. USA, *91:* 8969–8973, 1994.
15. Peifer, M., Berg, S., and Reynolds, A. B. A repeating amino acid motif shared by proteins with diverse cellular roles. Cell, *76:* 789–791, 1994.
16. Su, L-K., Johnson, K. A., Smith K. J., Hill, D. E., Vogelstein, B., and Kinzler, K. W. Association between wild-type and mutant *APC* gene products. Cancer Res., *53:* 2728–2731, 1993.
17. Joslyn, G., Richardson, D. S., White, R., and Alber, T. Dimer formation by an *N*-terminal coiled-coil in the APC protein. Proc. Natl. Acad. Sci. USA, *90:* 11109–11113, 1993.
18. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. Association of the *APC* gene product with β-catenin. Science (Washington DC), *262:* 1731–1733, 1993.

19. Su, L-K., Vogelstein, B., and Kinzler, K. W. Association of the APC tumor suppressor protein with catenins. Science (Washington DC), *262:* 1734–1737, 1993.
20. Smith, K. J., Levy, D. B., Maupin, P., Pollard, T. D., Vogelstein, B., and Kinzler, K. W. Wild-type but not mutant APC associates with the microtubule cytoskeleton. Cancer Res., *54:* 3672–3675, 1994.
21. Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B., and Polakis, P. The *APC* gene product associates with microtubules *in vivo* and promotes their assembly *in vitro*. Cancer Res., *54:* 3676–3681, 1994.
22. Fields, S., and Song, O-K. A novel genetic system to detect protein-protein interactions. Nature (Lond.), *340:* 245–246, 1989.
23. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. Cdi1, a human G1 and S phase protein phosphatase that associates with cdk2. Cell, *75:* 791–803, 1993.
24. Ruden, D. M., Ma, J., Li, Y., Wood, K., and Ptashne, M. Generating yeast transcriptional activators containing no yeast protein sequences. Nature (Lond.), *350:* 250–252, 1991.
25. Ron, D., and Dressler, H. pGSTag-A versatile bacterial expression plasmid for enzymatic labeling of recombinant proteins. Biotechniques, *13:* 866–869, 1992.
26. Powell, S. M., Petersen, G. M., Krush, A. J., Booker, S., Jen, J., Giardiello, F. M., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Molecular diagnosis of familial adenomatous polyposis. N. Engl. J. Med., *329:* 1982–1987, 1993.
27. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Wilson, J. K. V., Paraskeva, C., Petersen, G. M., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. The *APC* gene product in normal and tumor cells. Proc. Natl. Acad. Sci. USA, *90:* 2846–2850, 1993.
28. Pinkel, D., Landegent, J., Collins, C., Fuscoe, J., Segraves, R., Lucas, J., and Gray, J. Fluorecence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocation of chromosome 4. Proc. Natl. Acad. Sci. USA, *85:* 9138–9142, 1988.
29. Meltzer, P. S., Guan, X-Y., Burgess, A., and Trent, J. M. Micro-FISH: a novel strategy to identify cryptic chromosomal rearrangements. Nat. Genet., *1:* 24–28, 1992.
30. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. Basic local alignment search tool. J. Mol. Biol., *215:* 403–410, 1990.
31. Schuler, G. D., Altschul, S. F., and Lipman, D. J. A workbench for multiple alignment construction and analysis. Proteins Struct. Funct. Genet. *9:* 180–190, 1991.
32. Fattaey, A. R., Helin, K., Dembski, M. S., Dyson, N., Harlow, E., Vuosolo, G. A., Hanobik, M. G., Haskell, K. M., Oliff, A., Defeo-Jones, D., and Jones, R. E. Characterization of the retinoblastoma binding proteins RBP1 and RBP2. Oncogene, *8:* 3149–3156, 1993.
33. Kim, Y. W., Otterson, G. A., Kratzke, R. A., Coxon, A. B., and Kaye, F. J. Differential specificity for binding of retinoblastoma binding protein 2 to, R. B., p107, and TATA-binding protein. Mol. Cell. Biol., *14:* 7256–7264, 1994.