APCL, a Central Nervous System-specific Homologue of Adenomatous Polyposis Coli Tumor Suppressor, Binds to p53-binding Protein 2 and Translocates it to the Perinucleus

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ABSTRACT

APCL, a central nervous system-specific sequence homologue of the adenomatous polyposis coli tumor suppressor, can regulate the cytoplasmic level of β-catenin as the adenomatous polyposis coli tumor suppressor does, but its overall biological function remains unclear. Using a yeast two-hybrid system, we attempted to isolate proteins that might associate with the unique COOH-terminus of APCL. Among 166 cDNA clones isolated from a human fetal-brain cDNA library as candidates for interaction with APCL, 32 encoded parts of p53-binding protein 2 (53BP2), a molecule that interacts with p53 and Bcl2. An in vitro binding assay indicated that the Src-homology-3 domain and the ankyrin-repeat domain of 53BP2 were both required for binding to the COOH-terminus of APCL. Confocal microscopy showed that APCL and 53BP2 proteins were localized together in the perinuclei of normal mammalian cells, but this was not the case in cells that expressed truncated APCL and 53BP2 proteins. These findings suggested that binding of the COOH-terminus of APCL to 53BP2 regulates the cytoplasmic location of 53BP2. Because 53BP2 also interacts with p53 and Bcl2, we propose that binding of APCL might be involved in the p53/Bcl2-linked pathway of cell-cycle progression and cell death.

INTRODUCTION

APCL, a protein encoded on chromosome 19p13.3 that bears significant sequence homology to the APC1 tumor suppressor (1, 2) is expressed strongly and specifically in the central nervous system (3). The expression pattern indicates a specific role in proliferation and differentiation in the central nervous system, but the overall biological functions of APCL remain unclear. The heptad-repeat domain found in the APCL protein is well conserved in APC (45% of the amino acids are identical); therefore APCL likely also forms homo- or heterodimers (4). Moreover, because the Armadillo domain is also well conserved (76% identical), both proteins may interact with the same or similar molecular entities (5). The central portion of APCL consists of five copies of a 20-amino acid motif (FXVEXTPXCSFRXXSSLSSLS; Refs. 6, 7), and we showed earlier that through this domain, APCL can interact with β-catenin and deplete its intracellular concentration (3). Because the COOH-terminus of APC protein binds to EBI (8), microtubules (9), and hDLG (10), it appears that APC is involved in cell-cycle progression and/or growth control. However, because the COOH-terminus of the APCL protein bears little similarity to that of APC (only 13% identical amino acids), that part of the molecule can be presumed to interact with different proteins; if so, APCL would possess at least some functions distinct from those of APC.

To clarify those functions, we used a yeast two-hybrid system to search for proteins that might associate with the COOH-terminus of APCL. We found that this portion of the molecule indeed bound to a p53-binding protein, 53BP2. In vivo studies confirmed that this association regulated the cytoplasmic localization of 53BP2 by translocating it to the perinuclear region.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening. To generate a fusion protein containing a GAL4-binding domain, we excised a 2.6-kb NotI fragment (residues 1520–2303) of APCL cDNA, filled in the ends, and subcloned the fragment into a Smal site of pAS2-l vector (Clontech). After confirmation of the DNA sequences in the plasmid, we used it as “bait” to screen a human fetal-brain cDNA library using the pACT2 vector (Clontech). Briefly, the plasmids were transformed into Y190 yeast cells, and positive clones were selected on Trp(−), Leu(−), His(−) plates containing 25 mM 3-aminotriazole, and assayed for β-galactosidase activity by colony-lift filter assay. The positive clones were cotransformed with either the bait vector or the original pAS2-1 vector into yeast to confirm the interaction.

Quantitative β-Galactosidase Assays. A fusion plasmid containing the GAL4-binding domain and residues 458–1008 of 53BP2 was isolated from the yeast two-hybrid screening as the prey of the smallest fragment of 53BP2. Various fusion plasmids containing parts of 53BP2, i.e., 53BP2-A (residues 458–796), 53BP2-B (residues 756–921), 53BP2-C (residues 919–1008), or 53BP2-D (residues 756–1008), were subcloned into pACT2 vector as PCR-amplified fragments. A mutant 53BP2 plasmid, P53BP2-DM (W974K) was generated from pACT2-53BP2-D (residues 756–1008) by PCR-based site-directed mutagenesis. Missensecorporations of nucleotides during PCR amplification were checked by DNA sequencing. The bait and each 53BP2-fused plasmid were transformed into Y190 yeast cells, and five separate colonies were selected from the positive clones growing on Trp(−), Leu(−), His(−) plates containing 25 mM 3-aminotriazole. To confirm the interaction, positive clones were cotransformed into yeast with either the bait vector or the original pAS2-1 vector. We performed liquid culture assays for β-galactosidase in triplicate, using ONPG as a substrate according to the manufacturer’s instructions (Clontech).

Production of GST Fusion Proteins. cDNA fragments that contained parts of the coding sequence of 53BP2 were excised from the various pACT2-53BP2 plasmids by digestion with EcoRI and XhoI. Each fragment was ligated in-frame into pGEX5-2 (Pharmacia). Overnight cultures of XL1Blue MRF’, transformed with the plasmids encoding GST fusion proteins, were diluted and cultured with 0.1 mM isopropyl-1-thio-galactopyranoside for 6 h at 30°C. Cells were lysed by sonication in PBS containing 1% Triton X-100, and the lysates were clarified by centrifugation. Each GST-53BP2 fusion protein was purified on glutathione–agarose beads in PBS containing 1% Triton X-100.

In Vitro Binding Assay. We generated a [35S]Met-labeled COOH-terminus peptide of APCL from a template containing nucleotides 4120–6912 (stop) of the APCL cDNA sequence, using TNT rabbit reticulocyte lysate (Promega) according to the manufacturer’s instructions. The immobilized GST fusion proteins were mixed with the in vitro-translated APCL proteins in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA containing 0.1% Triton X-100.

Received 5/3/99; accepted 10/28/99.

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2 The abbreviations used are: APC, adenomatous polyposis coli; GST, glutathione-S-transferase.
X-100 and phenylmethylsulfonyl fluoride, incubated for 3 h at 4°C, then washed extensively four times with 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA. The beads were boiled in sample buffer and separated on 8% SDS PAGE gels. After electrophoresis, the gels were dried and exposed to X-ray film overnight at −80°C.

**Construction of a Mammalian Expression Vector for Epitope-tagged APCL and 53BP2.** Full-length APCL was inserted into pcDNA3.1(+)Myc-HisA vector (Invitrogen), and full-length 53BP2 was inserted to pFLAG-CMV vector (Kodak) in frame. Truncated APCL was generated using a 5.2-kb CpoI fragment containing residues 1–1675 of APCL, which lacked the 3’ region of interaction with 53BP2. This plasmid was subcloned into the EcoRV site of pcDNA3.1(+)Myc-HisB vector (Invitrogen) after blunting.

**Transfection to Mammalian Cells and Immunofluorescent Staining.** Cos7 and HeLa cells were cultured on coverslips and cotransfected by lipofection with 1 μg of pCMV-FLAG-53BP2 and 1 μg of either pCDNA3.1-APCL-Myc or pCDNA3.1-truncated-APCL-Myc, using Lipofectamine plus (Life Technologies, Inc.). Cells were fixed in 4% formaldehyde 24 h after transfection and dehydrated with 100% cold methanol for 10 min. After blocking in PBS containing 10% fetal bovine serum for 3 h, cells were treated with a mixture of mouse anti-Myc antibody (1:100; Santa Cruz Biochemistry, Inc.) and rabbit anti-FLAG antibody (1:100; ZYMED Laboratories, Inc.) in PBS for 1 h. The coverslips were washed in PBS three times for 10 min and then incubated in PBS for 1 h with a FITC-conjugated sheep antimouse antibody (1:100; Cappel) and a rhodamine-conjugated goat antirabbit antibody (1:100; Cappel). After the cells were washed four times in PBS, immunofluorescence was registered by means of a Zeiss LSM 410 confocal microscope.

**Western Blotting.** Cos7 cells were cultured and cotransfected with 1 μg of pCMV-FLAG-53BP2 and 1 μg of either pCDNA3.1-APCL-Myc or pCDNA3.1-truncated-APCL-Myc, using Lipofectamine plus (Life Technologies, Inc.). At 24 h after the transfection, the cells were harvested in lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 2% SDS). The lysed cell were sonicated and separated by 5% or 7% SDS-PAGE. Western

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**Fig. 1.** Schematic representation of the APCL protein structure compared with APC. The COOH-terminal region indicated in the schema (residues 1520–2303 of APCL) served as bait for yeast two-hybrid screening. aa, amino acid

**Fig. 2.** Interaction of APCL and 53BP2 in the yeast two-hybrid system. Quantitative β-galactosidase assays using several fusion plasmids of 53BP2 demonstrated that only 53BP2-D(756–1008), containing four ankyrin repeats and the SH3 domain, could specifically interact with the COOH-terminal region of APCL protein. In the mutant 53BP2-DM(756–1008), the most conserved residue of the SH3 domain was changed from W to K (W974K).
RESULTS

Interaction of APCL and 53BP2. To isolate human proteins capable of interacting with the COOH-terminus of the APCL protein, we screened a human fetal-brain cDNA library constructed with a vector containing a GAL4-activator domain. The bait for our yeast two-hybrid system was a fusion construct of the GAL4-binding domain and a NotI fragment (nucleotides 4561–7132) of APCL cDNA (Fig. 1). By screening 3.0 \( \times \) 10^7 yeast transformants, we isolated and sequenced 166 cDNA clones that interacted with APCL protein. Among them, 32 contained partial cDNA sequences of 53BP2; the clone containing the smallest fragment of 53BP2 included cDNA sequences corresponding to codons 458-1008. This region of 53BP2, which contains four adjacent ankyrin repeats and a Src-homology-3 (SH3) domain, is known to interact with p53 and Bcl2 (11, 12).

To determine which region(s) of 53BP2 are sufficient for binding to APCL, we constructed fusion plasmids containing a GAL4-activating domain and various parts of 53BP2. The 53BP2-B construct contained codons 756–921 (corresponding to four ankyrin repeats); 53BP2-C (codons 919-1008) contained the SH3 domain; and 53BP2-D (codons 756-1008) contained both the ankyrin repeats and the SH3 domain. To abolish the function of the SH3 domain, we constructed a mutant plasmid, 53BP2-DM (codons 756-1008; W974K) in which a tryptophan residue (W) at codon 974, the most conserved residue within the SH3 domain in 53BP2, was replaced with lysine (K). Quantitative b-galactosidase assays using these fusion plasmids demonstrated that only 53BP2-D could interact with the COOH-terminal region of the APCL protein (Fig. 2), indicating that the ankyrin-repeat region and the SH3 domain were both necessary for 53BP2 to interact with APCL.

In Vitro Binding Assay. To confirm the results of the b-galactosidase assays, several 53BP2 constructs were also tested in a GST pull-down assay. The in vitro-translated \([\text{35S}]\)-labeled COOH-terminus of APCL, coupled with the constructs indicated above each lane, was tested for binding to glutathione-agarose beads. A, GST-fusion proteins of 53BP2—GST-53BP2-D(756-1008), GST-53BP2-DM(756-1008 W974K), GST-53BP2-B(756-921), and GST-53BP2-C(919-1008)—were purified by glutathione-Sepharose, separated by SDS-PAGE, and stained with Coomassie blue. B, the COOH-terminal region of APCL protein specifically interacted with GST-53BP2-D(756-1008), but not GST alone, GST-53BP2-DM(756-1008 W974K), GST-53BP2-B(756-921), or GST-53BP2-C(919-1008).
hybrid system, the COOH-terminal region of APCL associated specifically with GST-53BP2, but not with GST alone, GST-53BP2-DM, GST-53BP2-B, or GST-53BP2-C (Fig. 3B).

Colocalization of Epitope-tagged APCL and 53BP2 in Mammalian Cells. To examine the subcellular locations of APCL and 53BP2, we cotransfected the two proteins into Cos7 or HeLa cells by means of expression plasmids encoding FLAG-tagged 53BP2 and either Myc-tagged full-length APCL or Myc-tagged truncated-APCL lacking the region that interacts with 53BP2. Western analysis of transfected cells revealed that FLAG-53BP2 was expressed as an ~150-kDa protein, APCL-Myc as a 260-kDa protein, and truncated APCL-Myc as a 210-kDa protein (Fig. 4).

Confocal microscopy revealed that full-length APCL was located mainly in the perinuclear region (Fig. 5A), but truncated APCL displayed a punctate staining pattern in the cytoplasm, which apparently was different from that of full-length APCL (Fig. 5B). 53BP2

Fig. 5. Regulation of the subcellular location of 53BP2 by APCL. A–C, Cos7 cells were transiently transfected with full-length APCL alone (A), truncated APCL alone (B), or 53BP2 alone (C). Full-length APCL and truncated APCL were detected with mouse anti-Myc antibody stained with FITC-conjugated sheep antimatouse antibody (green). 53BP2 was detected with rabbit anti-FLAG antibody stained with rhodamine-conjugated goat antirabbit antibody (red). Subcellular locations were visualized by immunofluorescence and confocal microscopy. D, colocalization of exogenous full-length APCL (green) and 53BP2 (red), appearing as yellow stain in the perinuclear regions of Cos7 cells. E, truncated APCL (t-APCL; green) was not detectable in the perinuclear pattern, nor was 53BP2 (red).
protein appeared in a punctate vesicular pattern in the cells transfected with 53BP2 alone (Fig. 5C), but in cells expressing both proteins, full-length APCL and 53BP2 were colocalized in the perinuclear region (Fig. 5D). However, in cells expressing truncated APCL and 53BP2, this was not the case: truncated 53BP2 displayed the same staining pattern as that in cells transfected with 53BP2 alone (Fig. 5E).

DISCUSSION

A brain-specific 2303-amino acid protein, APCL (3), possesses a high degree of sequence homology to APC in its NH₂-terminal and middle regions. However, its COOH-terminal region is different from that of APC, and because APCL is expressed specifically in brain, it is likely to play an important role in the central nervous system. To further elucidate the function(s) of APCL, we searched for proteins that might interact with its COOH-terminal region, using that portion of APCL as bait in a yeast two-hybrid screening system. This procedure detected interaction of 53BP2 with the COOH-terminal of APCL.

53BP2 binds wild-type p53 but not mutant p53 (13). Its amino acid sequence contains four ankyrin repeats and an SH3 domain in its COOH-terminus, and structural analysis has indicated that 53BP2 binds to wild-type p53 through those domains (12). 53BP2 also interacts with Bcl2, an inhibitor of apoptosis, and may therefore be involved in cell-cycle progression and cell death (11). Furthermore, 53BP2 seems to function in the signal transduction pathway by stimulating p53-mediated transcriptional activation (14). However, because this protein is present only in the cytoplasm (4), it probably cannot interact with nuclear p53. Hence, its functions remain unclear.

We have demonstrated here, using a yeast two-hybrid system and an in vitro binding assay, that the COOH-terminal region of APCL protein is able to interact with the four ankyrin repeats and the SH3 domain of 53BP2. Both APCL and 53BP2 appeared to be present in an insoluble fraction; they could be extracted only with buffers containing an ionic detergent such as SDS and sodium deoxycholate. We attempted to immunoprecipitate 53BP2 and APCL together in mammalian cells that overexpressed both proteins, but buffers containing such a strong detergent rendered our attempts unsuccessful.

Confocal microscopy revealed colocalization of APCL and 53BP2 in the perinuclear region of mammalian cells overexpressing both proteins, but 53BP2 was widely distributed over cytoplasmic structures in cells expressing 53BP2 alone or in cells expressing 53BP2 and truncated APCL proteins. These findings implied that the two proteins form a complex in vivo and that APCL can concentrate 53BP2 protein in the perinuclear region through attachment at its COOH-terminal region. When 53BP2 is overexpressed in mammalian cells, it also colocalizes with Bcl2 in the perinucleus (11). Therefore, when it is present in the perinuclear region, 53BP2 may function in the transduction pathway of Bcl2 or p53.

Regulation of the subcellular locations of components involved in various signaling pathways is crucial to effective initiation and maintenance of signaling cascades (15, 16). 53BP2 can promote p53 activity by stimulating p53-mediated transcriptional activation, thereby inducing cell-cycle arrest at the G2–M stage (11). These observations and the data presented here suggest that APCL might modulate the 53BP2-related signaling pathway by regulating the cytoplasmic localization of 53BP2, and also that APCL might be involved in mechanisms of cell-cycle progression and cell death linked to p53 and Bcl2. The clarification of this novel function of APCL may provide us new insights into the multiple functions of members of the APC family.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Misae Nishijima and Eiji Ouki.

REFERENCES


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