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Mammalian CAP Interacts With CAP, CAP2, and Actin

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Abstract We previously identified human CAP, a homolog of the yeast adenylyl cyclase–associated protein. Previous studies suggest that the N-terminal and C-terminal domains of CAP have distinct functions. We have explored the interactions of human CAP with various proteins. First, by performing yeast two-hybrid screens, we have identified peptides from several proteins that interact with the C-terminal and/or the N-terminal domains of human CAP. These peptides include regions derived from CAP and BAT3, a protein with unknown function. We have further shown that MBP fusions with these peptides can associate in vitro with the N-terminal or C-terminal domains of CAP fused to GST. Our observations indicate that CAP contains regions in both the N-terminal and C-terminal domains that are capable of interacting with each other or with themselves. Furthermore, we found that myc-epitope-tagged CAP coimmunoprecipitates with HA-epitope-tagged CAP from either yeast or mammalian cell extracts. Similar results demonstrate that human CAP can also interact with human CAP2. We also show that human CAP interacts with actin, both by the yeast two-hybrid test and by coimmunoprecipitation of epitope-tagged CAP from yeast or mammalian cell extracts. This interaction requires the C-terminal domain of CAP, but not the N-terminal domain. Thus CAP appears to be capable of interacting in vivo with other CAP molecules, CAP2, and actin. We also show that actin co-immunoprecipitates with HA-CAP2 from mammalian cell extracts.

Key words: adenylyl cyclase, BAT3, cytoskeleton, RAS, signaling, yeast

CAP was first identified as a protein that is associated with adenylyl cyclase in the budding yeast *Saccharomyces cerevisiae* [Field et al., 1988]. The gene encoding CAP was cloned and found to be allelic with mutations, *supC* and *SRV2*, that suppress the activated $RAS2^{val19}$ mutation [Field et al., 1990; Fedor-Chaiken et al., 1990]. Although CAP is required for the hyperactivation of adenylyl cyclase by $RAS2^{val19}$ in vivo, purified RAS is capable of activating adenylyl cyclase in crude membrane extracts derived from cells lacking CAP [Wang et al., 1992]. Deletion analysis has revealed that CAP has at least two distinct functional domains: the N-terminal domain is required for $RAS2^{val19}$ responsiveness of adenylyl cyclase, while the C-terminal domain is required for proper nutritional responses and cytoskeletal structure [Gerst et al., 1991; Vojtek et al., 1991]. Deletion of the C-terminal domain leads to several phenotypes including nutritional and temperature sensitivity, slow growth, abnormal cell morphology, random budding, and abnormal actin filament formation. Many of these phenotypes are also seen in conditional actin mutants or in yeasts that are deficient in profilin [Novick and Botstein, 1985; Haarer et al., 1990]. Also, overexpression of profilin suppresses these phenotypes in cap^- strains, although the normal budding pattern is only partially restored [Vojtek et al., 1991]. Overexpression of SNC1, a yeast homolog of the synaptobrevin family of synaptic vesicle–associated membrane proteins, also suppresses these phenotypes, but only in strains carrying the $RAS2^{val19}$ allele [Gerst et al., 1992]. The region separating the N-terminal and C-terminal domains of CAP consists of a proline-rich sequence that may represent another functional domain, although no phenotype has been observed in yeast lacking this domain. The functional relationships among the different domains of CAP are not understood.

CAP-related genes have been identified in the fission yeast *Schizosaccharomyces pombe* [Kawamukai et al., 1992], *Chlorohydra viridissima* [Fenger et al., 1994], and mammals [Matviw et al., 1992; Vojtek and Cooper, 1993;

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Zelicof et al., 1993; Yu et al., 1994]. Our previous studies suggest that at least some of the functional properties of CAP have been conserved between yeast and mammals [Matviw et al., 1992; Yu et al., 1994]. To further explore the function of mammalian CAP we sought to determine what proteins interact with CAP.

MATERIALS AND METHODS

Yeast Strains and Genetic Analysis

The yeast strains L40 (*MATa his3 trp1 leu2 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*) [Vojtek et al., 1993; Hollenberg et al., 1995] and SP1 (*MATa leu2 his3 ura3 trp1 ade8 can1*) [Kataoka et al., 1984] have been previously described. Yeast genetic and molecular biology techniques (i.e., growth, transformation, segregation analysis, etc.) were performed as described [Rose et al., 1990].

DNA Manipulation and Analysis

Procedures used for DNA manipulation and analysis (i.e., purification, restriction site mapping, electrophoresis, transformation, etc.) have been previously described [Sambrook et al., 1989]. The DNA sequence of both strands of sequenced clones was determined by a modified dideoxy chain-termination method [Sanger et al., 1977], using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied BioSystems, Foster City, CA). PCR reactions were performed as described [Matviw et al., 1992].

Plasmids

pCGH, used to construct and express HA-epitope-tagged proteins in NIH 3T3 cells, was constructed by inserting the coding sequence for the HA-epitope (MYPYDVPDYASLGGPMSTLD) [Field et al., 1988] into the *Xba*I site of the mammalian expression vector pCG [Tanaka and Herr, 1990]. pCIM, used to construct and express myc-epitope-tagged proteins in NIH 3T3 cells, was constructed by inserting the coding sequence for three tandem myc-epitopes (MEQKLISEEDLGEQKLISEEDLLEQKLISEEDLAA) [Evan et al., 1985] into the *NheI-EcoR*I sites of the mammalian expression vector pCI (Promega Corp., Madison, WI). pAD4H, used to construct and express HA-epitope-tagged proteins in *S. cerevisiae,* was constructed by replacing the 0.4 kb *SphI-Sal*I fragment from pAD4Δ [Ballester et al., 1989] with the 0.4 kb *Sph*I-*Sal*I fragment from pAD5 [Kawamukai et al., 1992]. pUAD6 used to construct and express myc-epitope-tagged proteins in *S. cerevisiae,* was kindly provided by Dr. Roymarie Ballester. pGEX-KG was used to construct and express GST fusion proteins [Guan and Dixon, 1991]. pGEX-NCAP encodes GST fused to the N-terminal domain of CAP (residues 1–228). pGEX-CCAP encodes GST fused to the C-terminal domain of CAP (residues 253–475). pMAL-c2 (New England Biolabs, Beverly, MA) was used to construct and express MBP fusion proteins. pMAL-Y1,2,3 was constructed by inserting cDNA fragments from pVP16-Y1,2,3 into pMAL-c2. pMALT, which expresses MBP, was constructed by removing the small Not I fragment from pMAL-Y1. The yeast two-hybrid expression vector pBTM116 contains the LexA coding sequence under the control of the *ADH1* promoter, the 2 μm origin of replication, and the *TRP1* gene [Bartel et al., 1993]. Plasmids that express LexA fusion proteins were generated by inserting DNA fragments into the polylinker of pBTM116 located 3′ to the LexA coding sequence. The yeast two-hybrid expression vector pVP16 contains the coding sequence of a nuclear localized VP16 acidic activation domain under the control of the *ADH1* promoter, the 2 μm origin of replication, and the *LEU2* gene [Vojtek et al., 1993; Hollenberg et al., 1995]. Plasmids that express VP16 fusion proteins were generated by inserting DNA fragments in the polylinker of pVP16.

Yeast Two-Hybrid Screen

The cDNA library used for the yeast two-hybrid screens contains short (400–700 bp) cDNA fragments constructed from 9.5 day mouse embryos and inserted into pVP16 [Vojtek et al., 1993; Hollenberg et al., 1995]. The *S. cerevisiae* strain L40 containing either pLexA-CCAP or pLexA-NCAP was transformed with the mouse embryo cDNA library using a high-efficiency transformation procedure [Schiestl and Gietz, 1989; Hill et al., 1991; Vojtek et al., 1993] to obtain an estimated 10^7 primary transformants for each screen. These transformants were grown in synthetic medium (YC-Trp-Ura-Leu-Lys) for 16 hr at 30°C to obtain efficient expression of the *HIS3* reporter gene. The transformants were then plated on selective synthetic plates (Yc-Trp-His-Ura-Leu-Lys) to select for cells that express HIS3. The plates were incubated for 3–5 days, and colonies were picked and tested for β-galactosidase activity by a filter assay [Breeden and

Nasmyth, 1985]. Segregation analysis of His^+LacZ^+ colonies yielded colonies that were dependent on the pLexA fusion plasmid for their His^+LacZ^+ phenotype. The pVP16 fusion plasmids were isolated from plasmid-dependent colonies, retransformed into L40 containing the pLexA fusion plasmid, and the assays for HIS3 and β-galactosidase activity were repeated.

Protein Analysis

Yeast expressing epitope-tagged proteins were grown to an $O.D._{600} = 1.0$, washed in H_2O, and resuspended in 200 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 mM PMSF, and leupeptin 1 μg/ml. Cells were broken by vortexing with glass beads, and the resulting extract was spun at 14,000 rpm in an Eppendorf centrifuge for 5 min to remove cell debris. Epitope-tagged proteins were transiently expressed in NIH 3T3 cells by electroporation of the appropriate plasmids [Chu et al., 1987; Baum et al., 1994]. After 24–48 hr the cells were briefly sonicated in extraction buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0) (400 μl/plate) and spun at 10,000 rpm for 5 min in an Eppendorf centrifuge to remove cell debris. Protein concentrations of the soluble extracts were determined using reagents from Bio-Rad Laboratories (Richmond, CA). Antibody preparation, immunoprecipitations, and Western blot analysis were performed as previously described [Harlow and Lane, 1988]. For immunoprecipitations, protein extracts were precleared with protein A Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) and then incubated with 12CA5 antibody in extraction buffer for 1 hr at 4°C. Protein A Sepharose was added to the antibody–antigen complex and incubated for 30 min at 4°C. The beads were washed four times with extraction buffer and resuspended in sample buffer.

In Vitro Binding Assays

Six micrograms of GST, GST-NCAP, or GST-CCAP was incubated with 50 μl glutathione-agarose beads (Sigma Chemical, St. Louis, MO) at 4°C for 2 hr. After sedimentation, the beads were incubated with 3% dry milk overnight to block nonspecific binding sites. The beads were washed with 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 20 mM Tris, pH 7.4. Seven point five micrograms of MBP or MBP fusions were incubated with the GST-fusion-glutathione-agarose beads in 60 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.005% NP40, 10% glycerol, 1.25% dry milk, 20 mM Tris, pH 7.4, at 4°C for 2 hr. The beads were washed four times with 80 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.01% NP40, 10% glycerol, 20 mM Tris, pH 7.4. The beads were then boiled in sample buffer and analyzed by Western blot analysis.

RESULTS

Yeast Two-Hybrid Screen

We used a yeast two-hybrid system to identify proteins that interact with the different domains of human CAP. We first constructed yeast expression vectors that express LexA fused to these domains, and then we screened for VP16 fusion proteins, encoded by a mouse embryo cDNA library, that interact with the LexA-CAP fusion proteins (see Materials and Methods). We isolated and characterized 14 clones from a screen for VP16 fusion proteins that interact with the C-terminal domain of CAP fused to LexA. Twelve of these clones encode overlapping regions derived from the N-terminal domain of the mouse CAP homolog. The smallest of these clones defines a minimal region of the N-terminal domain (residues 3–63) that is capable of interacting with the C-terminal domain of CAP. The other two clones overlap and they encode peptides with 90% identity to a region of human BAT3 (residues 246–360, 246–420), a protein with unknown function [Banerji et al., 1990].

We also isolated and characterized 24 clones from a screen for VP16 fusion proteins that interact with the N-terminal domain of CAP fused to LexA. Interestingly, one of these clones also encodes a region of BAT3 that overlaps with the two clones described above. Four other clones identified by this screen encode peptides derived from the N-terminal or C-terminal domains of CAP. We further found that VP16 fusions with the BAT3, N-terminal CAP, or C-terminal CAP peptides encoded by these clones also interact with either the C-terminal domain of CAP or full-length CAP fused to LexA (Fig. 1A). In contrast, the VP16 fusions encoded by the other clones we identified from this screen failed to interact with either the C-terminal domain or full-length CAP, suggesting that the sites in the N-terminal domain of CAP that interact with these proteins are not available in the full-length CAP protein (data not shown). Thus, these interactions may not be biologically relevant and we have not further characterized these clones. None of the VP16 fusion proteins

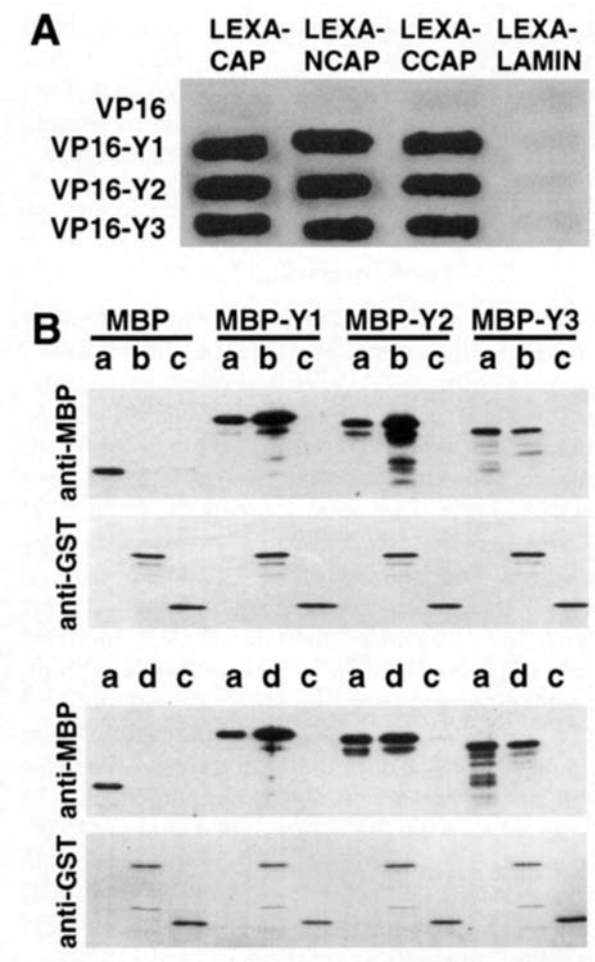

Fig. 1. CAP interacts with different peptides. **A:** The yeast two-hybrid system was used to select clones from a VP16-mouse cDNA fusion library encoding proteins that interact with the N-terminal (LexA-NCAP) or C-terminal (LexA-CCAP) domains of CAP fused to LexA. The VP16 fusions were tested for their ability to interact with LexA-LAMIN, LexA-NCAP, LexA-CCAP, or LexA-CAP by their ability to induce β-galactosidase activity in the yeast two-hybrid tester strain L40. Each patch represents an independent transformant containing plasmids expressing the indicated proteins. Y1 is 90% identical to residues 246–360 of human BAT3 (Genbank accession number M33519), Y2 is identical to residues 309–460 of mouse CAP (Genbank accession number L12367), Y3 is identical to residues 3–106 of mouse CAP. **B:** MBP fusions were tested for their abilities to interact in vitro with the N-terminal (GST-NCAP) or C-terminal (GST-CCAP) domains of CAP fused to GST. *Lanes a:* Six nanograms of MBP or the indicated MBP fusion. Seven point five micrograms of the indicated MBP fusion was incubated with glutathione beads bound to *(lanes b)* GST-NCAP, *(lanes c)* GST, or *(lanes d)* GST-CCAP (see Materials and Methods). Ten percent of the bound protein was analyzed by immunoblotting. MBP fusion proteins were detected using an MBP-specific polyclonal antibody (New England Biolabs). The GST, GST-NCAP, or GST-CCAP were detected using a GST-specific monoclonal antibody (Santa Cruz Biotech., Santa Cruz, CA). Antibodies were detected using the ECL chemiluminescent kit (Amersham, Arlington Heights, IL).

interact with LexA-Lamin, which was used as a negative control.

To further explore the interactions of the different domains of CAP with each other and with the BAT3-derived peptide, we performed in vitro binding assays using GST and MBP fusion proteins that were purified from bacteria expressing these proteins (Fig. 1B). Our results demonstrate that MBP fusions with the peptides derived from the N-terminal or C-terminal domains of CAP or BAT3 can interact with either the N-terminal or C-terminal domains of CAP fused to GST. Thus, CAP contains two distinct sequences that are capable of interacting with each other, or with the BAT3-derived peptide. The interactions between the different domains of CAP suggest that intermolecular and/or intramolecular interactions may occur between full-length CAP proteins.

CAP Interacts With CAP and CAP2

We next examined whether full-length CAP molecules can interact with each other in vivo. We found by the yeast two-hybrid test that VP16-CAP interacts with LexA-CAP (Fig. 2A). To further examine these interactions we constructed yeast and mammalian expression vectors that express either HA-epitope or myc-epitope-tagged CAP proteins. Using these vectors, we showed that myc-CAP co-immunoprecipitates with HA-tagged CAP, or the N-terminal or C-terminal domains of CAP, from extracts derived from yeast that express these epitope-tagged proteins, but not from extracts derived from control cells that express either HA-CAP or myc-CAP alone (Fig. 2B). We found similar results using extracts from NIH 3T3 cells that transiently express myc-CAP and/or HA-CAP (Fig. 2C).

We further examined whether CAP could interact with human CAP2. Our results using the yeast two-hybrid test indicate that LexA-CAP2 interacts with VP16-CAP (Fig. 3A). Furthermore, we demonstrated that myc-CAP2 coimmunoprecipitates with HA-CAP from extracts derived from NIH 3T3 cells that transiently express these proteins (Fig. 3B). Thus, CAP is capable of interacting with other CAP molecules, or with CAP2, in vivo.

CAP and CAP2 Interact With Actin

Previous results using the yeast two-hybrid test have suggested that yeast CAP interacts with yeast actin [Amberg et al., 1995; Freeman

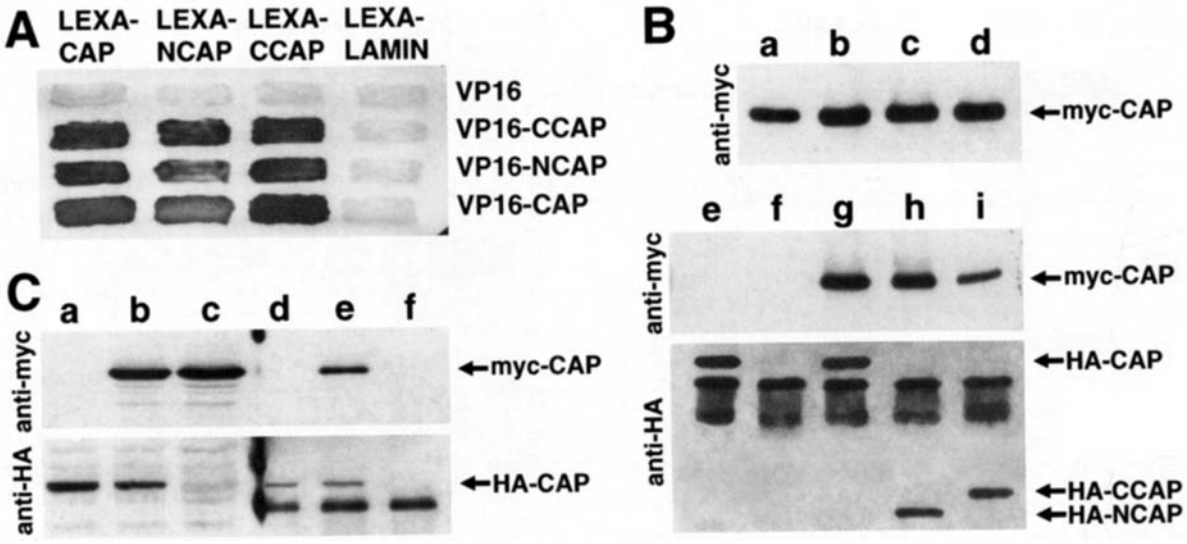

Fig. 2. CAP interacts with CAP. **A:** VP16 and VP16 fusions with the N-terminal (residues 1–228; VP16-NCAP) or C-terminal (residues 254–475; VP16-CCAP) domains of human CAP were tested for their abilities to interact with LexA fusions with the N-terminal (LexA-NCAP), C-terminal (LexA-CCAP), or full-length CAP (LexA-CAP) sequences by their abilities to induce β-galactosidase activity in the yeast two-hybrid tester strain L40. Each patch represents an independent yeast transformant expressing the indicated proteins. Yeast patches were stained for β-galactosidase activity [Breeden and Nasmyth, 1985]. **B:** An HA-specific monoclonal antibody 12CA5 (anti-HA) [Field et al., 1988] or a myc-specific monoclonal antibody 9E10 (anti-myc) [Evan et al., 1985] were used to probe Western blots of (**a**) extracts from yeast expressing myc-CAP alone, (**b**) myc-CAP and HA-CAP, (**c**) myc-CAP and HA-NCAP, or (**d**) myc-CAP and HA-CCAP, (**e**) 12CA5 immunoprecipitates of extracts from yeast expressing HA-CAP alone, (**f**) myc-CAP alone, (**g**) HA-CAP and myc-CAP, (**h**) HA-NCAP and myc-CAP, or (**i**) HA-CCAP and myc-CAP. **C:** 12CA5 (anti-HA) or 9E10 (anti-myc) were used to probe Western blots of (**a**) extracts from NIH 3T3 cells that transiently express HA-CAP alone, (**b**) HA-CAP and myc-CAP, or (**c**) myc-CAP alone, (**d**) 12CA5 immunoprecipitates of extracts from NIH 3T3 cells expressing HA-CAP alone, (**e**) HA-CAP and myc-CAP, or (**f**) myc-CAP alone. Antibodies which bound to the filters were detected using the ECL chemiluminescent kit (Amersham). The band present in all 12CA5 immunoprecipitates represents the heavy chain of 12CA5.

et al., 1995]. We sought to determine if these interactions have been conserved in mammals. Our results, using the yeast two-hybrid test, show that mammalian γ actin fused to VP16 can interact with a LexA-CAP fusion (Fig. 4A). Furthermore, we demonstrated that endogenous actin co-immunoprecipitates with CAP or the C-terminal domain of CAP, but not with the N-terminal domain of CAP, from extracts derived from either yeast or NIH 3T3 cells that express these HA-epitope-tagged proteins (Fig. 4B). Thus, an interaction between actin and human CAP can occur in yeast and mammalian cells, and this interaction requires the C-terminal domain, but not the N-terminal domain, of CAP. We also found that actin coimmunoprecipitates with HA-CAP2 from mammalian cell extracts (Fig. 4B).

DISCUSSION

We have identified VP16 fusions with several peptides, derived from various proteins, that interact with the C-terminal and/or N-terminal domains of human CAP. However, only peptides derived from CAP or BAT3 are capable of interacting with the full-length CAP protein. Interestingly, these peptides are capable of interacting with either the C-terminal or N-terminal domains of CAP, indicating that there are at least two similar binding sites in CAP. The interaction of full-length CAP with other CAP molecules indicates that CAP forms multimeric complexes in vivo. Our observation that both the N-terminal and C-terminal domains of CAP can interact with each other or with themselves further suggests that CAP may form large polymers. However, this may not occur since the interaction sites in the N-terminal domain may be unavailable in the full-length CAP protein, as appears to be the case for other peptides that we identified. Also, the formation of dimers may result in steric hindrance that precludes the association of additional CAP molecules. These possibilities remain to be fully explored.

We have previously found that CAP and CAP2 are expressed at different levels in different adult

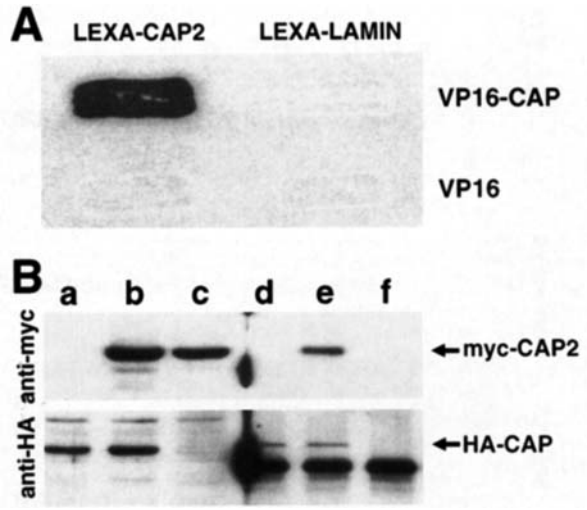

Fig. 3. CAP interacts with CAP2. **A:** VP16 or VP16 fused to human CAP were tested for their abilities to interact with LexA-CAP2 or LexA-lamin fusions by the yeast two-hybrid test. **B:** 12CA5 (anti-HA) or 9E10 (anti-myc) were used to probe Western blots of **(a)** extracts from NIH 3T3 cells that transiently express HA-CAP alone, **(b)** HA-CAP and myc-CAP2, or **(c)** myc-CAP2 alone, **(d)** 12CA5 immunoprecipitates of extracts from NIH 3T3 cells expressing HA-CAP alone, **(e)** HA-CAP and myc-CAP2, or **(f)** myc-CAP2 alone.

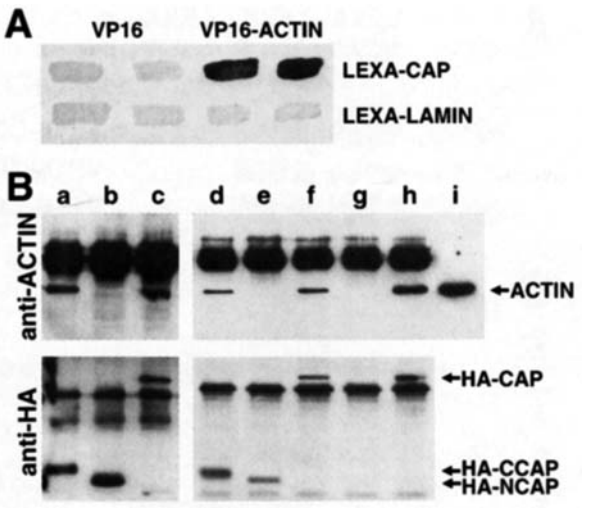

Fig. 4. CAP interacts with actin. **A:** VP16 and VP16 fusions with mammalian γ actin (VP16-ACTIN) were tested for their abilities to interact with LexA-CAP or LexA-LAMIN by their ability to induce β-galactosidase activity in the yeast two-hybrid tester strain L40. **B:** 12CA5 (anti-HA) or anti-actin monoclonal antibody (Boehringer Mannheim, Mannheim, Germany) were used to probe Western blots of **(a)** 12CA5 immunoprecipitates of extracts from yeast expressing HA-CCAP, **(b)** HA-NCAP, or **(c)** HA-CAP, **(d)** 12CA5 immunoprecipitates of extracts from NIH 3T3 cells expressing HA-CCAP, **(e)** HA-NCAP, **(f)** HA-CAP, **(g)** control vector (pCGH) alone, **(h)** HA-CAP2, or **(i)** 100 ng purified chicken skeletal muscle actin.

rat tissues, suggesting that they may have distinct roles [Swiston et al., 1995]. However, CAP and CAP2 are both present in many tissues. Our observations indicate that CAP and CAP2 can form a complex in vivo, suggesting that the formation of such complexes may regulate the roles of these proteins.

The functional significance of the interaction between CAP and the BAT3-derived peptide will require further investigation. There is no significant homology between BAT3 and any known protein sequence, and essentially nothing is known about the function of BAT3. However, there is a polyproline sequence in the middle of both BAT3 and CAP that suggests a structural and functional similarity. Also, BAT3 transcripts are present in a variety of adult rat tissues, but are found at very high levels in testis [Wang and Liew, 1994]. CAP is also ubiquitously expressed, but is found at high levels in testis, spleen, and lung [Vojtek and Cooper, 1993; Zelicof et al., 1993; Swiston et al., 1995]. Of the proteins we identified from our two-hybrid screen, the BAT3 peptide exhibits the strongest interaction with CAP in vitro. Furthermore, it is the only one we identified, other than CAP-derived peptides or actin, that can interact with the full-length CAP protein. Thus, this interaction may reflect a biologically significant relationship between these proteins.

Surprisingly, we did not identify clones encoding actin in our yeast two-hybrid screens. This may reflect the fact that the interaction between CAP and actin is relatively weak by the yeast two-hybrid test as compared with the interactions between CAP and the peptides we identified. The interaction of CAP with actin was suggested on the basis of previous genetic studies of yeast CAP [Vojtek et al., 1991; Amberg et al., 1995], and on the copurification of porcine CAP with actin [Gieselman and Mann, 1992]. The genetic studies indicate that yeast CAP is required for normal actin filament formation. More recent biochemical studies show that yeast CAP interacts with monomeric actin and prevents it from polymerizing in vitro [Freeman et al., 1995]. Since the identity between yeast and mammalian CAP proteins is only about 35%, the functional conservation of these proteins remains uncertain. Our observations demonstrate that human CAP and CAP2 also interact with actin; thus they support our previous studies suggesting that the functional properties of the C-terminal domains of CAP proteins have

been conserved between yeast and mammals [Matviw et al., 1992; Yu et al., 1994]. In yeast, CAP appears to be involved in both the regulation of adenylyl cyclase and actin filament formation. Thus, it may play a critical role in linking events of signal transduction and cytoskeletal reorganization. Perhaps mammalian CAP has a similar dual role.

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