Two Separate Functions Are Encoded by the Carboxyl-terminal Domains of the Yeast Cyclase-associated Protein and Its Mammalian Homologs

DIMERIZATION AND ACTIN BINDING

Cyclase-associated proteins were first identified as components of the RAS-activated cyclase complex. CAP consists of two functional domains separated by a proline-rich region. One domain, which localizes to the amino terminus, mediates RAS signaling through adenylyl cyclase, while a domain at the carboxyl terminus is involved in regulation of cell growth and morphogenesis. Recently, the carboxyl terminus of yeast CAP was shown to sequester actin, but whether this function has been conserved, and is the sole function of this domain, is unclear. Here, we demonstrate that the carboxyl-terminal domains of CAP and MCH1 homologs have two separate functions. We show that carboxyl-terminals of both yeast CAP and a mammalian CAP homolog, MCH1, bind to actin. We also show that this domain contains a signal for dimerization, allowing both CAP and MCH1 to form homodimers and heterodimers. The properties of actin binding and dimerization are mediated by separate regions on the carboxyl terminus; the last 27 amino acids of CAP being critical for actin binding. Finally, we present evidence that links a segment of the proline-rich region of CAP to its localization in yeast. Together, these results suggest that all three domains of CAP proteins are functional.

The yeast adenylyl cyclase-associated protein, CAP, was identified as a component of the RAS-activated cyclase complex. CAP consists of two functional domains separated by a proline-rich region. One domain, which localizes to the amino terminus, mediates RAS signaling through adenylyl cyclase, while a domain at the carboxyl terminus is involved in the regulation of cell growth and morphogenesis. Recently, the carboxyl terminus of yeast CAP was shown to sequester actin, but whether this function has been conserved, and is the sole function of this domain, is unclear. Here, we demonstrate that the carboxyl-terminal domains of CAP and MCH1 homologs have two separate functions. We show that carboxyl-terminals of both yeast CAP and a mammalian CAP homolog, MCH1, bind to actin. We also show that this domain contains a signal for dimerization, allowing both CAP and MCH1 to form homodimers and heterodimers. The properties of actin binding and dimerization are mediated by separate regions on the carboxyl terminus; the last 27 amino acids of CAP being critical for actin binding. Finally, we present evidence that links a segment of the proline-rich region of CAP to its localization in yeast. Together, these results suggest that all three domains of CAP proteins are functional.

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moter(3);pADH-CAP

pADH-CAP, which expresses antigen(HA) in yeast and mammals. This property is mediated by separate regions on the carboxyl terminus, suggesting that this region is both bifunctional and important for the regulation of the actin cytoskeleton in yeast and mammals.

**EXPERIMENTAL PROCEDURES**

**Media and Genetic Manipulations**

Yeast were grown in medium containing 2% glucose. Standard rich medium (YPD: yeast extract/Bactopeptone/dextrose), synthetic minimal medium (SC), and SC drop-out minimal medium, lacking an essential amino acid or nucleotide base, were prepared essentially as described by Rose et al. (18). Standard methods were used for the introduction of DNA into yeast and for the preparation of spheroplasts (18). R6 fibroblasts were cultured in Dulbecco's modified Eagle's (low glucose) medium (Life Technologies, Inc.) containing 10% bovine calf serum (Hyclone).

**DNA Manipulations**

DNA restriction endonucleases, Taq polymerase, and T4 DNA ligase were used as recommended by the suppliers (New England Biolabs and Promega). Molecular cloning techniques were performed as described by Sambrook et al. (19). The polymerase chain reaction (PCR) (20) and subcloning of PCR products were carried out as described previously (3).

**Plasmids**

Previously described vectors included: YCp50 (21); pTV3, a YEp-based multi-copy plasmid bearing the TRP1 selectable marker; pAD54, a YEp-based plasmid bearing the LEU2 marker and the ADH1 promoter (22); pAD54, a plasmid derived from pAD54 which contains an oligonucleotide encoding 22 amino acids of the influenza hemagglutinin antigen (HA) 5’ to a polycoding site; and pAD6, a plasmid derived from pAD43, which contains an oligonucleotide encoding 10 amino acids of the Myc epitope 5’ to the polycoding site. Other plasmids included: pAD-CAP, which expresses CAP under the control of the ADH1 promoter (3); pADH-CAPΔ4 and pADH-CAPΔ15, which express the COOH and NH₂ domains of CAP, respectively (3); pADH-CAPΔ17, which expresses a mutant CAP that contains the NH₂ and COOH domains, but lacks the proline-rich region (3); and pADH-MCH1 which expresses MCH1 under the control of the ADH1 promoter (14). Vectors used in the two-hybrid assay included: pCB6, which bears the sequence encoding the transactivating domain of GAL4 cloned upstream to a polycoding site; and pPC97, which encodes the DNA-binding domain of GAL4 cloned upstream to the polycoding site. These centromeric plasmids contain the TRP1 and LEU2 markers and were created by P. Chevray.

**Plasmid Construction**

Plasmid constructs made for this study were created using gene fragments synthesized in the PCR. Standard conditions for PCR were employed and included 25 cycles of denaturation (94 °C, 1.5 min), annealing (45 °C, 2.5 min), and extension (72 °C, 3.5 min). The resulting PCR products were gel purified and cloned into the pT7-Blue cloning vector (Novagen). The inserts were then subcloned into the appropriate yeast expression vectors and the resulting constructs were verified by restriction endonuclease analysis.

**Constructs Created for the Two-hybrid Assay—Oligonucleotides were used to create in-frame gene fusions between the sequences encoding either the DNA-binding domain or transactivating domain of GAL4 and genes of interest (e.g., CAP, CAP deletion mutants, and MCH1). The oligonucleotides used for gene amplification are listed in Table I. All GAL4-CAP fusions were created by subcloning the appropriate CAP fragment into the Sall and SacI sites of each plasmid. The list of plasmids created for this study is given in Table II. For the creation of the GAL4-CAP and GAL4-CAP (3) constructs, plasmid pUCAP (3) was used as template in the PCR reactions. For the creation of the GAL4-CAP (3) constructs, plasmid pADH-MCH1 was used as template. Protein expression was assayed by specific anti-CAP (1) and anti-MCH1 antisera (see below).

**Constructs Created for Immunoprecipitation Experiments—**To create epitope-tagged forms of CAP or CAP deletion mutants, CAP or mutant CAP genes were cloned downstream of, and in-frame to, the sequences encoding either the HA or Myc epitopes in plasmids pAD54 and pAD6, respectively. Single copy plasmids bearing the ADH1-HACAP or ADH1-HCAPΔ deletion mutants were created by subcloning BamHI fragments from pADH-HACAP or pADH-HCAPΔ plasmids into YCP50. Epitope-tagged forms of MCH1 and the MCH1AB/Δ4 deletion mutant were created in a similar fashion. Plasmids created for these experiments are listed in Table II. Protein expression was verified by functional testing in Δcap cells and by protein expression using anti-HA (2CA5), anti-Myc (9E10), or anti-MCH1 antisera.

**Yeast Strains**

For the two-hybrid assay, yeast strain Y153 (Mata gal4 gal80 his3 trp-902 ade2-101 ura3-52 leu2-3,112 Ura3::GalIacZ LYS2 ::Gal-I53) (23) was used. Δcap strains, SKN50 (Mata leu2 trp1 ade6 can1 1ra1::HIS3 cap::URA3) (6), and SKN32 and -34 (Mata leu2 trp1 ade6 can1 ura3 cap::HIS3) (1) were used for testing functional expression of both the Myc- and HA-tagged forms of MCH1 and CAP. A wild-type strain, SP1 (Mat1 leu2 ura3 trp1 ade6 can1 his3) (24), was also used.

**Antibodies**

A polyclonal antiserum against MCH1 was raised in rabbits using a MαE-MCH1 fusion protein as antigen. The gene fusion encoding the MαE-MCH1 protein was created by subcloning an EcoRI-Sall fragment of MCH1 into pMalC2 (New England Biolabs). This construct, pMalE-MCH1, was expressed in bacteria and MαE-MCH1 fusion pro-
tein was isolated by affinity chromatography, as recommended. We noticed that the fusion protein was truncated and had an apparent mobility of ~70 kDa in acrylamide gels. We calculated that the truncated MCH1 protein was ~35 kDa in size. After injection into rabbits and successive boosting, a polyclonal anti-MCH1 antiserum (number 30358) was obtained. This antiserum detects a single protein band of 60 kDa mobility in lysates prepared from rat fibroblasts and which could be competed for by the addition of exogenous MalE-MCH1 fusion protein to the immunoblot reaction (data not shown). Similarly, this antiserum could cross-react with any protein in wild-type cells (data not shown).

Immunoprecipitation and Immunoblot Analysis

The preparation of cell lysates for both immunoprecipitation and immunoblotting were performed as described by Couve and Gerst (8), with the exception that either 0.5 or 1.0% Triton X-100 was used as the final detergent concentration. Between 0.5 and 0.75 mg of total protein was incubated with 10 μl of affinity-purified anti-HA antibody (12CA5), 1.5 μl of anti-Myc (9E10) ascites fluid, or 3 μl of anti-MCH1 polyclonal antiserum in the immunoprecipitation reactions. Immunoprecipitation and immunodetection were performed as described (8).

Immunofluorescence Methods

To localize MCH1 in mammalian cells, Rat-6 fibroblasts were seeded at an initial density of 5 × 10^4 on pre-sterilized coverslips. After 24 h in medium containing 10% bovine calf serum, the coverslips were washed (×3) with PBS (phosphate-buffered saline) and fixed in a solution of paraformaldehyde (3%) and sucrose (3%) at room temperature, the coverslips were washed (×3) in PBS, 1% BSA for 3 min on ice. After incubation with permeabilization buffer, the coverslips were washed (×2) in PBS and treated for 5 min with 50 μl of Triton X-100 and 3 ml of MgCl2, for 3 min on ice. After incubation with permeabilization buffer, the coverslips were washed (×2) in PBS with bovine calf serum (PBS-BCS) for 15 min. After incubation with PBS-BCS, the coverslips were washed (×3) in PBS, 1% BSA (PBS containing 1% bovine serum albumin).

Primary and secondary antibody dilutions were prepared in PBS, 0.2% BSA. Anti-MCH1 polyclonal antiserum was diluted 1:1000; anti-vinculin antibody was diluted 1:200. After dilution, the antibodies were placed onto coverslips containing permeabilized Rat-6 cells and allowed to incubate for 60 min. After incubation with primary antibody at room temperature, the coverslips were washed (×3) in PBS for 10 min. The coverslips were then incubated with FITC-conjugated goat anti-rabbit antibody (1:500 dilution) or Texas Red-conjugated goat anti-mouse antibody (1:50 dilution) in PBS to quench the aldehyde fluorescence. Coverslips were washed (×2) with PBS and blocked with PBS containing 3% bovine calf serum (PBS-BCS) for 15 min. After incubation with PBS-BCS, the coverslips were rinsed (×3) in PBS, 1% BSA (PBS containing 1% bovine serum albumin).

TABLE II

Plasmid constructs created for this study

| Plasmid | Vector | Oligos used | Gene expressed |
|---------|--------|-------------|---------------|
| Two-hybrid | | | |
| pPC86-CAP | pPC86 | GJ6200|G151 | GAL4-CAP |
| pPC86-CAPΔ | | | |
| pPC86-CAPΔ20 | | | |
| pPC86-MCH1 | | | |
| pPC97-CAP | pPC97 | GJ6200|G151 | GAL4-CAP |
| pPC97-CAPΔ | | | |
| pPC97-CAPΔ20 | | | |
| Co-immunoprecipitation | | | |
| pADH-HACAP | pAD54 | GJ6200|G151 | HACAP |
| pADH-HACAPΔ4 | | | |
| pADH-HACAPΔ7 | | | |
| pADH-HACAPΔ11 | | | |
| pADH-HACAPΔ15 | | | |
| pADH-HAMCH1 | | | |
| pADH-HAMCH1Δ4 | | | |
| pADH-myCAP | pAD6 | GJ6200|G151 | myCAP |
| pADH-myCAPΔ4 | | | |
| pADH-myCAPΔ7 | | | |
| pADH-myCAPΔ11 | | | |
| pADH-myCAPΔ15 | | | |
| pADH-HMCH1 | | | |
| pADH-myMCH1Δ4 | | | |
| pTADH-HACAPΔ11 | pT3V | GJ6200|G151 | HACAP |
| YcpHACAP | YCp50 | GJ6200|G151 | HACAP |
| YcpHACAPΔ4 | | | |
| YcpHACAPΔ7 | | | |
| YcpHACAPΔ15 | | | |
| YcpHACAPΔ1 | | | |
| pMfe-MCH1 | pMfeC2 | A21/AAZ | MCH1 |
Yeast cells grown to log phase in synthetic minimal medium were harvested by centrifugation, washed, and fixed in a PBS solution containing 3% paraformaldehyde and 0.75% glutaraldehyde. Cells were harvested by centrifugation, washed, and fixed in a PBS solution containing 3% paraformaldehyde and 0.75% glutaraldehyde. Cells were washed, resuspended in a solution of 1% sodium metapolyedrate, and incubated for 45 min at 25 °C. The fixed cells were treated with 50 mM NH₄Cl, dehydrated sequentially in ethanol, and embedded in Lowicryl K4M resin. Fixed cells were sectioned and mounted on 200-mesh grids stained with 5% uranyl acetate in 25% ethanol for 40 min and quickly washed with a solution of 5 mM lead citrate in 0.01 M NaOH. Electron microscopy was performed on a Hitachi TEM 7000.

Immunogold labeling of thin-sectioned yeast was performed using 20-nm Protein A-gold (E-Y Laboratories). Prior to uranyl acetate lead citrate staining, grids containing thin sections were incubated with PBST (PBS containing 0.05% Tween) for 15 min before incubation in PBST and 1% BSA (PBST-BSA). The grids were then incubated for 2 h at room temperature with anti-CAP antiserum (1:500 dilution) in PBST-BSA. Following incubation, the grids were washed 5 times with PBST and further incubated with Protein A-gold diluted 1:50 in PBST-BSA for 1 h at room temperature. The grids were washed, as described above, and fixed with 0.25% glutaraldehyde in PBS. Staining of the sections with uranyl acetate and lead citrate, and electron microscopy was performed as described above.

RESULTS

CAP and MCH1 Dimerize and Form Both Homologous and Heterologous Interactions: Two-hybrid System—In order to demonstrate protein-protein interactions between cyclase-associated proteins and other cellular factors, we created gene fusions between GAL4 and CAP, or MCH1, for use in the two-hybrid assay (see "Experimental Procedures"). This assay has been used reliably to demonstrate interactions between proteins that form tight complexes with one another, such as the retinoblastoma susceptibility gene product (Rb) and protein phosphatase 1α (PP1α) (23).

During testing of GAL4-MCH1 and GAL4-CAP fusions in yeast bearing Gal4-inducible reporter elements (e.g. GAL-LaC and GAL-HIS3), we noticed that the cyclase-associated proteins form productive interactions with each other. These interactions resulted both in the expression of lacZ reporter activity, as well as, conferring survival in the presence of a metabolic inhibitor, 3-aminotriazole (3-AT). Survival of the metabolic block is directly related to metabolic inhibitor, 3-aminotriazole (3-AT). Survival of the metabolic block is directly related to metabolic inhibitor, 3-aminotriazole (3-AT). Survival of the metabolic block is directly related to metabolic inhibitor, 3-aminotriazole (3-AT). Survival of the metabolic block is directly related to metabolic inhibitor, 3-aminotriazole (3-AT).

Gene fusions created between MCH1 and regions encoding either the Gal4 transactivating domain (TA) or the Gal4 DNA-binding domain (DB) yielded activities described in A and B. In contrast, co-expression of Gal4-CAP fusion proteins gave little to no lacZ reporter activity, but was able to confer weak growth in the presence of the metabolic block (Fig. 1, A and B). None of the gene fusions yielded lacZ activity, or growth on 3-AT-containing medium, when expressed individually in cells (Fig. 1). These results imply that MCH1 can form specific protein-protein interactions either with itself or with CAP. This idea is supported by the subsequent screening of a mammalian cDNA library in the two-hybrid system. Among proteins capable of interacting with MCH1, we were able to isolate human CAP (12) from a Gal4-cDNA fusion library prepared from HeLa cell cDNAs (data not shown).

We have previously shown that CAP is bifunctional. A domain corresponding to the amino terminus of CAP associates directly with adenyl cyclase (27) and mediates activation of the enzyme by RAS (1, 3). A second domain which corresponds to the carboxyl terminus of CAP is involved in growth control and cellular morphology (3, 4). Finally, a region of unknown function separates the amino- and carboxyl-terminal domains. This region bears the proline-rich stretch of residues that may constitute one or more SH3 binding domains (16, 17).

In order to determine which region is responsible for dimerization, we tested various deletion mutants of CAP (fused with either the Gal4 DNA-binding or transactivating domain) for activity in the two-hybrid assay. These deletion mutants have been previously characterized by us with respect to both protein expression and phenotypic suppression in Δcap yeast (3). Gene fusions that conferred growth on medium lacking histidine and cellular viability in the presence of 3-AT included: TA-CAP, TA-MCH1, DB-CAP, and DB-MCH1. The Gal4 DB domain alone is given as DB. Plasmids were maintained by growth on synthetic double selective medium (-Trp, -Leu). To select for protein-protein interactions that lead to growth in the absence of histidine, and in the presence of a metabolic block for histidine synthesis, patches were replica plated onto triple selective medium containing 25 mM 3AT (-Leu,-Trp,-His -3AT), and allowed to grow (3 days). Yeast bearing plasmids which express Gal4-Rb and Gal4-PP1α (DB)Rb and (TA)PP1α, respectively were used as positive control, A, assay of lacZ reporter activity. Cell extracts were made from strains expressing Gal4TA and Gal4DB fusion proteins described in A and were assayed for β-galactosidase activity (see "Experimental Procedures"). C, regions of cyclase-associated proteins required for interaction, as assayed by lacZ reporter activity. Cell extracts were made from strains expressing different CAP and MCH1 fusions with the TA or DB domains of Gal4, and included the CAP deletion mutant, CAP(12-260)-696-526 (N + C). Units of β-galactosidase activity are expressed in nanomoles of o-nitrophenolgalactoside deaved/mg of protein/h. The average of two separate experiments are given. Error bars indicate the standard error of the mean.

FIG. 1. MCH1 and CAP form homodimers and heterodimers. A, two-hybrid assay for growth on medium lacking histidine. Plasmids expressing full-length MCH1 and CAP fused with either the transactivating domain (TA) or the DNA-binding domain (DB) of Gal4 were tested in Y153 cells. Expressed proteins included: (TA)CAP, (TA)MCH1, (DB)CAP, and (DB)MCH1. The Gal4 DB domain alone is given as DB. Plasmids were maintained by growth on synthetic double selective medium (-Trp, -Leu). To select for protein-protein interactions that lead to growth in the absence of histidine, and in the presence of a metabolic block for histidine synthesis, patches were replica plated onto triple selective medium containing 25 mM 3AT (-Leu,-Trp,-His -3AT), and allowed to grow (3 days). Yeast bearing plasmids which express Gal4-Rb and Gal4-PP1α (DB)Rb and (TA)PP1α, respectively were used as positive control, A, assay of lacZ reporter activity. Cell extracts were made from strains expressing Gal4TA and Gal4DB fusion proteins described in A and were assayed for β-galactosidase activity (see "Experimental Procedures"). C, regions of cyclase-associated proteins required for interaction, as assayed by lacZ reporter activity. Cell extracts were made from strains expressing different CAP and MCH1 fusions with the TA or DB domains of Gal4, and included the CAP deletion mutant, CAP(12-260)-696-526 (N + C). Units of β-galactosidase activity are expressed in nanomoles of o-nitrophenolgalactoside deaved/mg of protein/h. The average of two separate experiments are given. Error bars indicate the standard error of the mean.
shown). This fusion protein expresses a deletion mutant of CAP that lacks the proline-rich region (CAPΔ7), but is fully functional and can suppress the loss of both the amino- and carboxyl-terminals of CAP (3). Moreover, the CAP1–169/369–526 protein was found to interact tightly with itself (data not shown).

We performed quantitative analysis of lacZ reporter activity in order to verify the interactions described above for MCH1 and CAP1–169/369–526, as well as, for CAP1–169/369–526 and itself. We were able to reproducibly detect reporter activities in cells expressing Gal4 fusions with CAP and MCH1, MCH1 and CAP1–169/369–526, and CAP1–169/369–526 and itself (Fig. 1C).

However, we were unable to detect reporter activity in cells expressing fusions between Gal4 and the middle domain of CAP or MCH1 (data not shown). Finally, none of these described fusion proteins induced enzyme activity, when expressed individually (data not shown).

These results indicate that MCH1 is likely to form a tight physical complex with itself and can form heteromeric complexes with yeast CAP. In addition, the proline-rich domain of CAP does not appear essential for this interaction.

Immunoprecipitation Experiments—Since the two-hybrid assay is known to yield both false positive, as well as, false negative results we decided to confirm our findings using a different approach. In order to demonstrate any physical interactions between the CAP and MCH1 proteins, and to define the regions required for these interactions, we employed a co-immunoprecipitation approach.

First, to demonstrate MCH1 dimerization, we expressed both HA-tagged and Myc-tagged forms of MCH1 in yeast, and performed immunoprecipitations (IPs) with the anti-HA antibody in cell lysates. The results show that MCH1 is specifically co-precipitated along with with HA-MCH1 (Fig. 2A). Thus, as predicted, MCH1 forms homologous associations in yeast. We next determined whether MCH1 and CAP form heterologous associations, as also predicted by the two-hybrid experiments. As shown in Fig. 2B, native CAP can be detected along with HA-MCH1 in immune complexes precipitated by the anti-HA antibody. Moreover, the co-precipitation of CAP is eliminated when the IPs are performed in the presence of excess HA peptide. Thus, we can verify that MCH1 interacts heterologically with CAP.

Although CAP does not interact well with itself in the two-hybrid system (for reasons that remain unknown), we tested whether we could detect a physical association between the proteins using co-immunoprecipitation. We expressed both HA-tagged CAP and Myc-tagged CAP in wild-type yeast and performed IPs on cell lysates using the anti-HA antibody. In contrast to the previous results from the two-hybrid system, we could clearly detect the presence of Myc-CAP in these immune complexes and could block its detection by the addition of excess HA peptide to the IP reaction (Fig. 2C). Therefore, CAP, like MCH1, interacts tightly with itself.

In order to identify those domains of CAP which mediate dimerization, we used cells expressing both HA-CAP and Myc-tagged CAP deletion mutants in a second series of experiments. Both protein expression and function of the individual domains were verified using 1-cap cells (3 and data not shown). We found that HA-tagged CAP co-precipitates with Myc-tagged CAP1–169/369–526 as well as, with the carboxyl terminus of CAP (CAP291–526) (Fig. 3A). In contrast, HA-CAP does not co-precipitate with either the amino terminus of CAP (CAP1–192) or with the middle proline-rich domain (Fig. 3A). Thus, it appears that dimerization is mediated through the carboxyl terminus.

We also examined the interaction of HA-CAP1–169/369–526 with Myc-tagged CAP1–169/369–526 and other Myc-tagged CAP domains. We observed that deletion mutants lacking the middle proline-rich region interact tightly with each other and with both the carboxyl-terminal domain (CAP14; CAP291–526) and the amino-terminal domain (CAP15; CAP1–192) (Fig. 3B). Thus, the CAP1–169/369–526 mutant interacts more tightly with the two functional domains of CAP than does native CAP. This suggests that the middle proline-rich domain could, potentially, act to inhibit the ability of CAP to form dimers.

Because we were able to demonstrate a direct interaction between the carboxyl terminus of CAP and either full-length CAP or CAP1–169/369–526 we examined whether this domain could mediate protein dimerization by itself. In co-IP experiments, we found that the tagged carboxyl-terminal domain (CAP291–526) could, in fact, co-precipitate with itself (Fig. 3C).
Precipitates the tagged amino-terminal domain (CAP 1–192) (Fig. 3). In addition, we were able to show that this domain could also be precipitated by co-immunoprecipitation.

Extracts from yeast co-expressing either the HA-tagged carboxyl-terminal domain of CAP, or the carboxyl-terminal domain of MCH1, were subjected to immunoprecipitation. Immune complexes were resolved on SDS-acrylamide gels and transferred to nylon membranes. Immunoblots were probed with an anti-actin monoclonal antibody (20 μg/ml) (Boehringer Mannheim). B, the carboxyl-terminal domain of CAP and MCH1 binds actin. Lysates from Δcap cells expressing various Myc-tagged domains of CAP, or the carboxyl-terminal domain of MCH1, were subjected to immunoprecipitation. Immunoblots were incubated with an anti-actin monoclonal antibody (20 μg/ml). Antigen detection was performed by ECL chemiluminescent assay.

In order to demonstrate whether the actin-binding function of the carboxyl terminus of yeast CAP is conserved, we performed IP experiments to assay for the presence of actin in precipitated complexes formed with either epitope-tagged MCH1 or CAP, as control. As shown in Fig. 4A, a protein of ~46 kDa can be detected in protein complexes formed in the presence of HA-tagged CAP or HA-tagged MCH1, using an anti-actin antibody. Moreover, this same interaction occurs with the CAP deletion mutant, CAP1–169/369–526, as well as with the carboxyl terminus of CAP or MCH1 (MCH1277–474) (Fig. 4B). In contrast, neither the amino terminus, nor the middle proline-rich domain of CAP, have this activity, although expression of these proteins was verified by immunoblot analysis (Fig. 4B and data not shown). Thus, we conclude that both CAP and its mammalian homolog, MCH1, are actin-binding proteins and that the domain required for this interaction localizes to the carboxyl-terminal domains. Thus, the carboxyl terminus of both CAP and MCH1 have two distinct functions: dimerization and actin binding.

Separation of the Dimerization and Actin-binding Functions—In order to demonstrate whether the two functions of the carboxyl terminus are mediated by the same domain, we examined whether a specific CAP mutant, CAP1–498 (CAPΔ111), can dimerize and bind to actin. This mutant lacks the last 27 amino acids of the protein and is unable to confer phenotypic
and actin binding are likely to be mediated by separate domains on CAP and expressed only epitope-tagged native or mutant CAP. These experiments were performed in cells lacking endogenous suppression of the loss of the carboxyl terminus of CAP (3). We first examined whether CAP 1–498 is capable of dimerization, either with native CAP (Fig. 5A) or with itself (Fig. 5B). We found that CAP 1–498 is fully capable of undergoing dimerization with itself or with native CAP (Fig. 5, A and B). Thus, although CAP 1–498 is incapable of restoring normal growth and morphology (3), its ability to undergo dimerization is unaffected.

We next examined whether CAP 1–498 binds actin. Co-precipitation experiments (Fig. 5, A and B) demonstrate that unlike native CAP (Figs. 4A and 5A), CAP 1–498 is unable to bind to actin (Fig. 5B), suggesting that the last 27 amino acids of CAP participate in the actin-binding function. Moreover, since CAP 1–498 dimerizes with either itself or native CAP (Fig. 5, A and B), it would seem that the ability of CAP to dimerize is not dependent upon actin binding. Thus, protein dimerization and actin binding are likely to be mediated by separate domains on the carboxyl terminus. Importantly, these results also imply that the actin-binding function of the carboxyl terminus is directly related to its ability to suppress the growth and morphological phenotypes which occur upon CAP disruption.

Finally, we noticed that precipitation of the CAP-CAP 1–498 heterodimer (immunoprecipitated with the anti-Myc antibody) brought down significant levels of actin (Fig. 5A), unlike immunoprecipitation of the CAP 1–498 homodimer (Fig. 5B). Thus, the native CAP protein present in the heterodimer is still able to interact with actin.

Removal of the Proline-rich Domain of CAP Alters Its Localization in Yeast—We have also examined the requirements for the cellular localization of CAP in yeast, using thin-section microscopy and immunogold labeling to detect the presence of CAP deletion mutants. Native CAP protein was found to localize primarily to the cytosol in wild-type cells (data not shown). Similarly, Δcap cells expressing the carboxyl terminus of CAP (CAP Δ4; CAP 291–526) are also labeled in the cytosol (Fig. 6, panel 2). This construct does not bear the polyproline stretch of residues found in CAP (residues 277–285), but does bear the second proline-rich segment (residues 354–361). In contrast, the CAP 1–169/369–526 deletion mutant and were not seen with any of the other CAP mutants. Thus, it appears that removal of the proline-rich middle domain of CAP results in the aggregation and mislocalization of the protein. Moreover, it appears that the second proline-rich segment of the middle domain (residues 354–361) may be necessary for normal localization.

Localization of MCH1 in Mammalian Cells—Since MCH1 is an actin-binding protein, we have assayed for the localization of MCH1 in mammalian cells, as well as, its ability to localize with cellular actin. First, we fixed Rat-6 fibroblasts and labeled them with either a polyclonal anti-MCH1 antiserum (number 30358) or phalloidin, as described under "Experimental Procedures." Through the use of fluorescence microscopy, we deter-
separate and specific protein-protein interactions conferred by CAP, MCH1, and other cellular components. Using two-hybrid signed to reveal possible protein-protein interactions between yeast and mammalian CAPs, we have undertaken studies defined that MCH1 localizes primarily to the cytosol, but partially strong labeling is also observed in the region of the cell defined areas that are likely to be points of cellular adhesion. In freshly attached fibroblasts, however, it is clear that MCH1 does not localize to the same regions. Thus, MCH1 is unlikely to be a component of focal contacts.

**DISCUSSION**

To help resolve the functions of the carboxyl terminus of yeast and mammalian CAPs, we have undertaken studies designed to reveal possible protein-protein interactions between CAP, MCH1, and other cellular components. Using two-hybrid and co-immunoprecipitation studies, we can demonstrate two separate and specific protein-protein interactions conferred by this domain: protein dimerization and actin binding. Importantly, these functions appear to be mediated by separate regions of the carboxyl terminus and are conserved evolutionarily. Thus, the carboxyl terminus of these proteins must possess separate signals for dimerization and actin sequestration. A structure-function diagram which summarizes the results obtained for yeast CAP is presented in Fig. 8.

We have shown that the carboxyl terminus (COOH) of the cyclase-associated proteins is sufficient, by itself, to confer dimerization. Nevertheless, we cannot rule out participation of the amino terminus (NH$_2$) in the process. In fact, co-immunoprecipitation studies using mutant CAP proteins demonstrate that the carboxyl terminus can interact either with itself, or with the amino terminus. Thus, sequences which confer dimerization must be present in both domains. Analysis of the CAP protein, using the PredictProtein algorithm, reveals that the secondary structure of the amino-terminal (−160 amino acids) is composed almost entirely of α-helical segments. In contrast, the carboxyl terminus (also −160 amino acids) is composed principally from β-strands. It is unclear, at present, how these domains promote their protein-protein interactions.

Our results do suggest some interesting possibilities, however. First, if the proline-rich region acts as a flexible hinge, then the NH$_2$ and COOH domains of the CAP monomer might interact with one another. This could, potentially, represent a state of the protein that might be unable to interact with either adenyl cyclase or actin. However, there is no data to support the idea that CAP exists as monomers in cells. In fact, cell lysates prepared and electrophoresed under nondenaturing conditions show that CAP cannot be resolved as either a monomer or dimer and was unable to enter even low percentage acrylamide gels. Thus, CAP appears to exist solely as a component of a high molecular weight protein complex in wild-type cells; perhaps, the adenyl cyclase complex, as previously demonstrated (1, 5).

A more likely alternative is that CAP proteins are present in their dimeric form, as suggested from this work. Because the carboxyl terminus may interact with both domains, dimerization might occur in either a parallel (COOH::COOH) or anti-parallel fashion (NH$_2$::NH$_2$). The significance of this is unclear, but does allude to the possibility that different and, perhaps, functionally distinct CAP protein-protein complexes could result from changes in the orientation of the dimer.

Since disruption of the CAP gene leads to drastic alterations in the actin cytoskeleton and as profilin is capable of suppressing those phenotypes, it is highly probable that CAP is involved in regulation of the cytoskeleton (4). Recent studies have supported this contention by showing that the carboxyl terminus of...
CAP binds directly to actin (28, 29). Here, we have not only verified those experiments, but also show that the carboxyl terminus of a mammalian CAP homolog mediates a similar function. Thus, the role of CAP in cytoskeletal regulation is likely to have been conserved evolutionarily. This second finding of ours was predicted by an earlier study which demonstrated actin binding to a putative porcine CAP homolog (11). Thus, CAP and its mammalian homologs (MCH proteins) constitute a novel family of actin-binding proteins.

Yeast CAP was found to inhibit actin polymerization and to associate with actin monomer in a 1:1 stoichiometric relationship (28). On the basis of this, we suggest that each carboxyl-terminal of the CAP homodimer may bind a single actin molecule. Thus, one might predict that the molecular mass of the actin-bound CAP homodimer would be on the order of 240 kDa (70 kDa for each molecule of CAP and 46 kDa for each molecule of actin). Since CAP is a component of a large molecular weight complex in yeast, this cannot be adequately verified in vivo. However, the heterologous expression of MCH1 in yeast results in the formation of a ~200 kDa protein complex that contains MCH1, when the cell lysates are prepared and electrophoresed under non-denaturing conditions. Moreover, MCH1, like CAP, was not found to exist as a monomer. This lends credence to the idea that CAP (or MCH proteins) exist primarily in an actin-bound state and could explain why the size of the yeast RAS-responsive adenyl cyclase complex decreases by ~250 kDa in Δcap cells (from 890 to 610 kDa) (5).

Our results demonstrate that the functions of the carboxy terminus of CAP are distinct and separable. A CAP deletion mutant, CAP1–498, was found to dimerize with itself, or with native CAP, but was unable to bind to actin. Thus, the last 27 amino acids of CAP are involved in actin binding, but are not necessary for dimerization. Moreover, actin binding is not a pre-requisite for CAP dimerization, although, it is unclear whether dimerization is required for actin binding. There is no sequence present in the last 27 amino acids of CAP that predicts an actin-binding function. Furthermore, sequence comparison between CAP, MCH1, and other actin-binding proteins, using the MACAW protein alignment program, revealed no obvious conserved motifs that might be implicated in this function (data not shown). Therefore, it is unclear whether this region binds actin directly.

The function for the third, middle proline-rich domain of CAP has, until recently, remained elusive. We have found that removal of the proline-rich region of CAP does not impair protein dimerization and, in contrast, may even enhance it. This hypothesis is borne out by studies which demonstrate that a mutant, but fully functional CAP protein that lacks the polyproline stretch (CAP1–169/369–526) (3), yields higher reporter activity than native CAP, when used in the two-hybrid system (Fig. 1C), and forms mislocalized protein aggregates when expressed alone in Δcap yeast (Fig. 6). This suggests that the proline-rich region of CAP may act to restrict oligomerization and that CAP protein aggregation occurs in its absence. A second possible function for this region may be to properly localize CAP in yeast. Electron micrographic studies reveal a cytosolic distribution for both native CAP protein and the carboxyl terminus (which lacks the polyproline PPPPPPAPP stretch (residues 277–285), but bears the PPPRPKKP stretch (residues 354–361)). However, the CAP1–169/369–526 mutant, which lacks both proline-rich segments, resides in electron-dense aggregates. Thus, it would seem likely that residues 354–361 are important for CAP localization, but not for dimerization. Nearly identical localization results have been shown recently by Freeman et al. (31) with these and other CAP deletion mutants, using immunofluorescence. Thus, the results from both studies (this study and Ref. 31) argue that the middle domain of CAP acts as a possible localization signal. This is not altogether surprising, due to the resemblance of the proline-rich segments to known SH3-binding domains (16). These motifs are known to aid in protein localization (32), particularly with components of the actin cytoskeleton. In fact, the SH3 domain of Abp1, a yeast actin-binding protein, was shown to interact with the proline-rich region of CAP, suggesting that Abp1 might play a significant role in CAP function (31). However, the deletion of Abp1 does not lead to Δcap phenotypes and the localization of CAP is unaltered in abp1 cells. Thus, it is unlikely that Abp1, by itself, mediates CAP localization and function.

We are continuing to examine the relationship between profilin and CAP in yeast. Profilin was isolated as a suppressor in high copy of the deletion of the carboxyl-terminal domain of CAP (4) and it has been suggested that both the actin- and phosphoinositide-binding functions of profilin are relevant toward ameliorating Δcap defects. Our results confirm the idea that the ability of profilin to suppress the disruption of CAP is due, at least in part, to its actin-binding function. We have examined whether there is a direct relationship between profilin and CAP, using both two-hybrid and co-immunoprecipitation methodologies. However, we have been unable to demonstrate any physical interaction between these proteins and, as of yet, there is no firm reason to believe that profilin acts downstream of CAP. It may be that profilin and CAP act independently of one another, but share overlapping functions.

This study raises many interesting possibilities regarding both the structure and function of the CAP protein complex. For example, is CAP always present in its dimeric form in the cyclase complex? If so, perhaps CAP acts as a scaffold which holds the cyclase-CAP-actin complex together and localizes it to the actin cytoskeleton. Additionally, the presence of multiple SH3-binding domains in the dimer might allow for the association of different SH3-containing proteins, like those involved in the regulation of RAS function (i.e. Cdc25 or Ira1/Ira2). A more important question is whether the actin-binding function is under regulatory control, perhaps, by the RAS signaling pathway? If so, then RAS effector function and cytoskeletal control may be tightly linked and could be modulated, in turn, by other physiological signals which impact upon these processes (i.e. pheromone signaling and mating, bud site initiation, and bud emergence). Additional studies will be required to address these open questions.

Acknowledgments—We thank Dr. Stephen Elledge for the generous gift of the yeast strains and plasmids of the two-hybrid system and Dr. Patrick Brennwald for the 9E10 anti-Myc antibody. In addition, we thank Drs. Robert Krauss, Sandra Masur, and Scott Henderson for helpful discussions and useful advice.

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