A Cytoskeletal Localizing Domain in the Cyclase-associated Protein, CAP/Srv2p, Regulates Access to a Distant SH3-binding Site*

(Received for publication, February 26, 1999, and in revised form, April 27, 1999)

Jong Yu‡, Christopher Wang‡, Stephen J. Palmieri§, Brian K. Haarer§, and Jeffrey Field‡¶

From the ‡Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 and the ¶Section of Cell and Developmental Biology, University of Texas, Austin, Texas 78712

In the yeast, *Saccharomyces cerevisiae*, adenylyl cyclase consists of a 200-kDa catalytic subunit (CYR1) and a 70-kDa subunit (CAP/SRV2). CAP/Srv2p assists the small G protein Ras to activate adenylyl cyclase. CAP also regulates the cytoskeleton through an actin sequence and is directed to cortical actin patches by a proline-rich SH3-binding site (P2). In this report we analyze the role of the actin cytoskeleton in Ras/cAMP signaling. Two alleles of CAP, L16P(Srv2) and R19T (SupC), first isolated in genetic screens for mutants that attenuate cAMP levels, reduced adenylyl cyclase binding, and cortical actin patch localization. A third mutation, L27F, also failed to localize but showed no loss of either cAMP signaling or adenylyl cyclase binding. However, all three N-terminal mutations reduced CAP-CAP multimer formation and SH3 domain binding, although the SH3-binding site is about 350 amino acids away. Finally, disruption of the actin cytoskeleton with latrunculin-A did not affect the cAMP phenotypes of the hyperactive Ras2Val19 allele. These data identify a novel region of CAP that controls access to the SH3-binding site and demonstrate that cytoskeletal localization of CAP or an intact cytoskeleton per se is not necessary for cAMP signaling.

The Ras proto-oncogene is one of the most frequently activated genes in tumors. It is widely conserved in evolution, and many homologs are interchangeable in genetic and biochemical assays. All Ras proteins are small G proteins, active when bound to GTP but inactive when bound to GDP. Oncogenic Ras mutants contain amino acid substitutions that reduce the GTPase activity resulting in elevated levels of GTP-bound Ras. Upon activation by GTP binding, Ras activates downstream proteins known as effectors (for reviews see Refs. 1–3). Ras signals can be divided into two categories, those controlling transcription through mitogen-activated protein kinase cascades and those controlling cell morphology and the actin cytoskeleton through alternate pathways (4). In most organisms Ras regulates the actin cytoskeleton through subsequent activation of the Rho family of G proteins to initiate a cascade of small GTPases (5–7). Ras has not, however, been shown to regulate actin in the budding yeast *Saccharomyces cerevisiae*, although Ras regulates morphogenesis in the fission yeast *Schizosaccharomyces pombe* through a GTPase cascade to the Rho family member Cdc42sp (8).

Adenylyl cyclase, which is the Ras effector in the yeast *S. cerevisiae*, was the first protein identified that interacted with a Ras ortholog (9–11). Ras regulates adenylyl cyclase by activating its only known activity, catalysis of ATP into the second messenger cAMP. The catalytic subunit is ~220 kDa in size and can be divided into five functional regions: (i) a N-terminal region of no known function, (ii) a leucine-rich repeat Ras-binding region (12, 13), (iii) a spacer region, (iv) a catalytic domain, and (v) C-terminal CAP/Srv2-binding region (14–17). Ras activates the catalytic activity through direct binding to the second region. The role of CAP in cAMP signaling is to facilitate Ras activation of adenylyl cyclase, perhaps by binding the farnesyl group at the Ras C terminus (18).

CAP (also known as Srv2p) was first identified as a 70-kDa protein that co-purified with adenylyl cyclase; it was also independently isolated twice in genetic screens for genes required for Ras signaling (14, 15, 19). Loss of function mutants of CAP reduce cAMP levels and suppress the phenotypes of the hyperactive Ras2Val19 allele. The adenylyl cyclase interacting region of CAP was mapped genetically and later biochemically to the N terminus of CAP. This region binds the C terminus of adenylyl cyclase, perhaps through a coiled-coil interaction (16, 17, 20).

In addition to Ras signaling, CAP maintains the cytoskeleton. Loss of CAP causes an abnormal yeast morphology and disrupts the actin cytoskeleton. The actin-associated phenotypes are partially restored by overexpression of the C terminus of CAP or the actin monomer-binding protein, profilin (20–22). This C-terminal region binds actin monomers in *vitro* (23). Furthermore, the first mammalian homolog CAP1, or ASP-56, was isolated as an actin monomer-binding protein (24). All other homologs, where tested, also bind actin through the C-terminal region (25–27).

All CAP homologs contain a centrally located proline-rich region. In yeast, this domain can be subdivided into two regions, the P1 and P2 sites. The P1 site, found in almost all homologs, contains a 10–12-amino acid stretch composed almost entirely of prolines, and its function remains unknown. The P2 region contains a consensus SH3-binding motif (PXXP), binds SH3 domains in *vitro*, and is required to direct CAP to cortical actin patches (28). Abp1p (actin filament-binding protein 1) can bind the P2 region through its SH3 domain and is a strong candidate for a targeting protein because *abp1* strains have reduced levels of CAP in cortical actin patches (29).

Localization of CAP to the actin cytoskeleton suggests that it may translocate adenylyl cyclase to cortical patches, hence serving as an adapter protein. However, deletion studies found that the N-terminal region of CAP was sufficient to restore heat shock sensitivity to a cap::Ras2Val19 strain, whereas the C-terminal region of CAP restored only the actin-
related phenotypes (20). Moreover, no actin phenotypes have been observed in any Ras mutants. Together, these observations suggest that CAP regulates cAMP and actin independently. However, studies to date either inferred the role of CAP by deleting the CAP-binding site on adenyl cyclase or tested CAP mutations in Ras2Val19 strains (15, 30). Ras2Val19 strains harbor an activated Ras. Wild type cells require Cdc25p to activate Ras by GTP exchange (31–35). Thus, if CAP or the actin cytoskeleton was required for Cdc25p to activate Ras, no effects on cAMP signaling would have been observed in the previous studies.

Another conserved region of CAP is found in the extreme N terminus within the adenyl cyclase-binding site (see Fig. 1). The reason for the conservation of the adenyl cyclase-binding site is not clear, because mammalian homologs do not associate with particulate adenyl cyclase in platelets, where both are highly expressed. In addition, the CAP-binding domain of adenyl cyclase is not conserved in mammalian cyclases.(2) Recently, this region was found to associate with CAP itself, suggesting that a multi-subunit complex may form consisting of two or more molecules of CAP and actin (26, 27).

In this report we demonstrate that the adenyl cyclase-binding region is required for subcellular localization of CAP to actin cortical patches, but the localization function can be separated from the adenyl cyclase binding function and cAMP signaling through effector mutants. Evidence is presented that this region is required for CAP self-association and access to the SH3-binding site to direct subcellular localization. Because this domain is found in all CAP homologs, its function is likely to be conserved. Furthermore, we demonstrate that disruption of the actin cytoskeleton does not block Ras coupling to adenyl cyclase.

**EXPERIMENTAL PROCEDURES**

**Construction of CAP Plasmids**—For YCP50-CAP plasmids, CAP was amplified using the forward primer 5′-CATCAAGAAGCGTTCAGGAGGAG-3′ and reverse primer 5′-CCTGTTATACCTAGGAGATCC′ from genomic DNA of wild type yeast (SP1) and yeast containing mutant CAP (Y019 and TKR1–2LS). The amplified fragments contained 1279 base pairs upstream of the start codon and 390 base pairs downstream of the stop codon and were cloned into the HindIII and BamHI sites of YCP50. Mutagenesis was performed by the unique restriction site elimination method (36). The CAP-L27F point mutant was generated by the primer 5′-ACTGCAAGATTGGAATGTCACCATC-3′, which introducing from template provided by Roger Tsien (pRSET-S65T) (37) using PfuMutagen (Stratagene) (La Jolla, CA). Mutations were verified by sequencing.

**Biochemical Methods**—For GFP immunoprecipitations and GST-ABP1-SH3 coprecipitations, yeast (DDY817 with appropriate plasmids) were grown in 1 l of synthetic medium containing glucose, galactose, and appropriate supplements to select for plasmids for 4–5 days at 25 °C. Protein extracts were prepared as described previously (38). Monomeric CAP antibody (CAP 100) described in Ref. 16 was used to detect proteins in most Western blots (polyclonal antibody 154 was used in Fig. 6A). GFP immunoprecipitations were carried out using 2 μl of a 1:10 diluted polyclonal antibody (CLONTECH), 300 μl of cytosolic fraction extract, 600 μl of buffer C, and 30 μl of a 50% suspension of protein A-agarose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. Coprecipitations were carried out using purified GST-ABP1-SH3, 300 μl of cytosolic fraction extract, 600 μl of buffer C (38), and 30 μl of a 50% suspension of glutathione-agarose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. The beads were washed three times with 1 ml of buffer C containing 1% Lubrol and 0.5 mM NaCl, transferred to new tubes, and washed once with buffer C for both reactions. Glucose feeding and cAMP measurements were performed as described previously (15, 39).

**Strains, Manipulations, and Growth Media**—The S. cerevisiae strains used are described in Refs. 14, 15, and 28. The genotype of JF7C is MATa ura3-52 leu2-3,112 his3. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose), YPDGalactose (1% yeast extract, 2% peptone, 1% dextrose, 1% galactose), or synthetic medium (0.67% yeast nitrogen base on base, 2% dextrose, and 1% dextrose) containing the appropriate auxotrophic supplements. Yeast genetic manipulations were performed as described (40). Heat shock sensitivity was assayed by growing yeast (JF5037 with appropriate plasmids) pads on a YPD plate followed by replica plating and placing the plates at 55 °C for 0–20 min. The plates were then incubated at 30 °C for 2–3 days. Latrunculin-A was purchased from Molecular Probes (Eugene, OR).

**Two-Hybrid System**—CAP was amplified from genomic DNA of wild type strain (SP1) and mutant yeast (Y019 and TKR1–2LS) using forward primer 5′-CGGGGCGAATTCGTCGAGACCTCGAGCAAAAAAGCCATC-3′ and reverse primer 5′-CGCGCGGAATTCATGCCTGACTCTAAGTACACA-3′ amplifying from template provided by Roger Tsien (pRSET-S65T) (37) using PfuMutagen (Stratagene) (La Jolla, CA). HindIII digestion and 5′-CGCTGTATACCTAAGGGATCC-3′ primers were used to amplify the first 262 amino acids, which encode the first 262 amino acids, and 5′-CGCTAGCGGCCGCTTA-3′, which encodes the amino acids 1821–2026 of CYR1 in pJG4–5. The system used was a modified version of the Brent system (41). The following plasmids and vectors were used: pSH18–34, URA3 LexA-operator/LacZ reporter; pEG202, HIS3 plasmid containing LexA fusion protein (‘bait’); pG4–5, TRP1 plasmid containing expression library or specific interacting protein; pSH17–4, HIS3 plasmid, LexA fused to the activation domain of LexA, positive control for activation. The system was twofold overexpressed in the yeast strain EGY48, β-Galactosidase activity was measured as described (42).

**GFP-CAP Localization and Phalloidin Staining**—Yeast cells (JF7C with appropriate plasmids) were grown on YPDGalactose plates for 5–6 days to induce production of the GFP-CAP fusions. Cells were then incubated in YPDGalactose liquid medium for 3 h at 30 °C. 600 μl of saturated culture were washed twice with water and suspended in 20 μl of 20% polylysine and mounted onto a slide to view live yeast cells. 2.5 ml of saturated culture was fixed with formaldehyde for 4% for 10 min. Cells were suspended in 0.1 mM potassium phosphate with 10 mM ethylenediamine for 10 min, washed once, and suspended in 500 μl of 0.1% potassium phosphate. Rhodamine-phalloidin (Sigma) was incubated to a concentration of 1 μg/ml for 1 h at 25 °C. Cells were washed twice with phosphate-buffered saline, suspended in 20 μl of 2% polylysine, and mounted onto a slide. Cells were viewed under a Zeiss Axiopt microscope equipped for epifluorescence microscopy or confocal microscopy and photographed with Kodak Ektachrome II 400 film.

**RESULTS AND DISCUSSION**

**Mutations in the N Termminus of CAP Prevent Localization to the Actin Cytoskeleton**—CAP was isolated two ways, first as an adenyl cyclase-binding protein and second in genetic screens for mutations that suppress the activated Ras2Val19 allele (14, 15). To determine the mutations in the original isolates, we performed chain reaction amplified DNA from the two alleles, which we will refer to as the SupC and Srv2 alleles, and sequenced the products. We found that the SupC allele had a Thr for Arg substitution at amino acid 19 (R19T) and the Srv2 allele had a substitution of Pro for Leu at amino acid 16 (L16P) (Fig. 1). These were the only mutations found in the gene, and the same mutations were found in multiple independent reac-

---

2 N. Freeman and J. Field, unpublished observations.

3 The abbreviation used is: GFP, green fluorescent protein.
Modulation of Localization and SH3 Binding in CAP/Srv2p

Fig. 1. Map of the yeast CAP gene and sequences of the mutants used in this study.

| Mutant | Amino acid change | Codon change |
|--------|------------------|-------------|
| srv2   | L16P             | CTA ▶ CCA   |
| supC   | R19T             | AGG ▶ ACG   |
| L27F   | TTA ▶ TTC        |
| P2     | P355A, P358A     | CCA ▶ GCA   |

After we established the GFP system, we tested localization of the N-terminal mutants. At this time we also constructed L27F using site-specific mutagenesis to change Leu-27, an amino acid conserved in the mammalian homologs, to Phe. The L27F, R19T, and L16P fusions all restored growth on rich medium when expressed in a cap strain (data not shown). The N-terminal mutants were also constructed alone and together with the ΔP2 mutation, because this site is required for subcellular targeting to actin cortical patches. We found that each of the three N-terminal mutations, R19T, L16P, and L27F, failed to localize correctly (Fig. 2). Double mutants with the P2 site and each of the three N-terminal mutations also failed to localize to cortical actin patches (data not shown). Thus, the GFP fusions confirmed that a new targeting region is located in the N terminus of CAP.

Actin Cortical Patch Localization Can Be Uncoupled from cAMP Signaling—The discovery that the adenylyl cyclase-binding region of CAP was required for cytoskeletal localization prompted us to determine whether CAP localization to the actin cytoskeleton was required for cAMP signaling. To do this we introduced the CAP mutants into cap yeast and measured cAMP signaling three different ways. First we used a genetic assay that reflects cAMP levels (Fig. 3), second we measured cAMP directly in cells (Fig. 4), and third we measured binding of CAP to adenylyl cyclase (Fig. 5). The genetic assay is performed by testing the sensitivity to heat shock in a RAS2Val19 strain. The RAS2Val19 mutation is analogous to some activating mutations in human cancers and reduces the intrinsic GTPase activity of Ras (44). The increase in cAMP levels causes cells to be sensitive to brief heat shock treatments at 55 °C. Mutations in CAP lower cAMP levels and cause cells to be resistant to this treatment (14, 15). We found that, as expected from their isolation in a genetic screen, the SupC (R19T) and Srv2 (L16P) mutants failed to restore sensitivity. The L27F mutation, like wild type CAP, was still sensitive, suggesting that it did not reduce the cAMP levels in the Ras2Val19 strain. The P2 mutation alone did not alter CAMP responses by itself, nor did it alter the responses of any of the other mutants (Fig. 3; data not shown for P2).

Because the genetic assay depends on the Ras2Val19 allele, it only measures Ras coupling to adenylyl cyclase; therefore, it is largely independent of upstream inputs through Cdc25p. The effect of upstream inputs to Ras can be measured by using a glucose feeding assay. When cells are grown and then starved for several hours they have very low levels of cAMP. Addition of glucose to the starvation buffer stimulates a rapid rise in cAMP levels. The stimulation is transient with cAMP levels returning almost to starting levels in about 5 min. Unlike the genetic assay above, the glucose feeding assay requires Cdc25p.
found that the L16P mutation completely abolished glucose stimulation of cAMP (Fig. 4). Cells expressing the R19T mutation still responded by elevating their cAMP levels, but the peak was much smaller than cells expressing wild type CAP. The L27F mutant responded normally in the assay. These data suggest that subcellular localization of CAP does not play a role in cAMP signaling.

However, as discussed above, the P2 region is also required for proper localization. To determine whether a single localization signal is sufficient for proper cAMP responses, we mutated this site alone and in each of the original mutants. We found that mutating this site alone and in combination with any of the three N-terminal mutants did not influence cAMP responses. The L16P mutant still showed no response to glucose, the R19T mutant still had a partial response, and the L27F mutant still behaved the same as wild type (Fig. 4). Thus we conclude that cAMP signaling does not require targeting of CAP to actin cortical patches.

We used two different assays to measure adenylyl cyclase binding to CAP, the yeast two-hybrid assay, and adenylyl cyclase coimmunoprecipitations (45). In the two-hybrid assay, each mutant bound adenylyl cyclase when tested for the Leu2 reporter or tested on 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (X-gal) plates for the blue color caused by the \(\beta\)-galactosidase reporter (data not shown). However, when \(\beta\)-galactosidase activity was measured using a more quantitative enzyme assay, the L16P mutant interacted weakly, and the R19T mutant interacted to about 50% of wild type CAP, whereas the L27F mutant interacted as strongly as wild type CAP (Fig. 5). In all cases, reporter activity was galactose-dependent because the trap expression was driven by a galactose-inducible reporter. A similar level of interaction was observed when we used a CAP antibody to immunoprecipitate adenylyl cyclase activity from yeast extracts. All bound adenylyl cyclase as compared with negative controls. However, in all experiments there was a significant reduction in the binding by the L16P and R19T mutants compared with wild type (5–10-fold less); L27F bound more adenylyl cyclase than the other mutants in two of three experiments, although binding was usually somewhat lower (about 2-fold) than wild type. However, variations in the total activity in the cell extracts made quantitative comparisons between the different mutants unreliable with this assay (data not shown). In summary, adenylyl cyclase binding, unlike subcellular localization, correlated with cAMP signaling.

All N-terminal Mutants Reduce SH3 Binding and CAP-CAP Association—The proline-rich SH3-binding site is required to localize CAP to the actin cytoskeleton. To determine whether the N-terminal mutations, located about 350 amino acids away from the P2 site, affected SH3 binding, we performed GST pull down experiments with the SH3 domain of Abp1 to precipitate CAP from crude yeast extracts (Fig. 6). The precipitates were then probed with a CAP antibody on a Western blot to assess SH3 binding. The P2 mutant did not bind in this assay (Fig. 6A). Interestingly, we found that all three N-terminal mutants severely impaired in binding Abp1-SH3 (Fig. 6B). This suggests that their failure to localize was due to reduced association with Abp1 in vitro.

CAP can associate with itself, perhaps to form dimers (26, 27). Sequences in both the N terminus and C terminus may mediate the interaction. To determine whether the mutant CAPs could associate with themselves, we expressed the mutants from two plasmids, GFP-CAP and untagged CAP, in a cap

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** L16P, R19T, L27F, and \(\Delta_{P2}\), three mutations in the N terminus of CAP and a mutation in SH3-binding site, respectively, prevent localization to actin cortical patches. Fusion proteins between GFP and the indicated proteins were constructed as described under “Experimental Procedures” and then tested for localization in the cap strain JF7C. The cells in A were fixed and co-stained with rhodamine-labeled phalloidin and photographed using a confocal microscope. The cells in B were observed and photographed live using a Zeiss Axioskop microscope equipped for epifluorescence.

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** The CAP N-terminal mutants L16P and R19T, but not L27F suppress the heat shock sensitivity of a Ras2\(^{Val19}\) strain. The cap::Ras2\(^{Val19}\) strain JFSK37 (SKN37) was transformed with the indicated plasmids and tested for heat shock sensitivity by incubating at 55°C for the indicated times immediately after replica plating. Cells were allowed to recover for 3 days and then photographed.
We then prepared extracts, immunoprecipitated the fusion proteins with a GFP antibody, and probed Western blots with a CAP antibody. Because the GFP protein is about 20 kDa larger than CAP, we could easily distinguish the two proteins on Western blots. We found that the wild type GFP-CAP fusion protein readily coprecipitated the untagged protein, which is seen as a 70-kDa band in Fig. 7. However, each of the three point mutants, L16P, R19T, and L27F, precipitated much less untagged protein. Because none of these proteins localized correctly, multimerization may be essential for directing CAP to actin cortical patches.

The experiments described so far have not directly addressed the role of actin in cAMP signaling but just the actin interacting regions of CAP. Thus, if actin were required for cAMP signaling through CAP-independent mechanisms we would have not detected it. Actin is encoded by Act1, which is an essential gene, so its role cannot be tested through knockout technology. Recently, however, the drug latrunculin-A was found to rapidly disassemble the yeast cytoskeleton by depolymerizing actin. In vitro, latrunculin-A binds actin monomers and prevents them from assembling into filaments (46). In vivo, the effects are rapid but are readily reversed when the drug is washed away. Thus, cells can be tested in the presence of the drug, and the drug can then be washed away to allow cells to grow. To determine whether the actin cytoskeleton mediated Ras interaction with adenylyl cyclase, we incubated wild type and Ras2Val19 cells with latrunculin-A, performed heat shock experiments, and then determined whether the Ras2Val19 cells became resistant to heat shock when they were treated with latrunculin-A (Fig. 8). We found no differences in viability upon drug treatment. That is, Ras2Val19 cells were still heat shock-sensitive when tested in the presence of latrunculin-A. This suggests that F-actin itself is not required for Ras to activate adenylyl cyclase.

We present evidence that the N terminus of CAP/Srv2p is required for localization to actin cortical patches. Three mutations within the extreme N terminus produced proteins that failed to localize to actin cortical patches as determined by immunofluorescence and GFP fusion tagging. This domain overlapped with the adenylyl cyclase-binding site but could be distinguished from it because one mutant that failed to localize, L27F, still bound adenylyl cyclase normally and maintained a wild type cAMP signaling system. Previous studies demonstrated that a proline-rich SH3-binding site, P2, is required for protein readily coprecipitated the untagged protein, which is seen as a 70-kDa band in Fig. 7. However, each of the three point mutants, L16P, R19T, and L27F, precipitated much less untagged protein. Because none of these proteins localized correctly, multimerization may be essential for directing CAP to actin cortical patches.

The experiments described so far have not directly addressed the role of actin in cAMP signaling but just the actin interacting regions of CAP. Thus, if actin were required for cAMP signaling through CAP-independent mechanisms we would have not detected it. Actin is encoded by Act1, which is an essential gene, so its role cannot be tested through knockout technology. Recently, however, the drug latrunculin-A was found to rapidly disassemble the yeast cytoskeleton by depolymerizing actin. In vitro, latrunculin-A binds actin monomers and prevents them from assembling into filaments (46). In vivo, the effects are rapid but are readily reversed when the drug is washed away. Thus, cells can be tested in the presence of the drug, and the drug can then be washed away to allow cells to grow. To determine whether the actin cytoskeleton mediated Ras interaction with adenylyl cyclase, we incubated wild type and Ras2Val19 cells with latrunculin-A, performed heat shock experiments, and then determined whether the Ras2Val19 cells became resistant to heat shock when they were treated with latrunculin-A (Fig. 8). We found no differences in viability upon drug treatment. That is, Ras2Val19 cells were still heat shock-sensitive when tested in the presence of latrunculin-A. This suggests that F-actin itself is not required for Ras to activate adenylyl cyclase.

We present evidence that the N terminus of CAP/Srv2p is required for localization to actin cortical patches. Three mutations within the extreme N terminus produced proteins that failed to localize to actin cortical patches as determined by immunofluorescence and GFP fusion tagging. This domain overlapped with the adenylyl cyclase-binding site but could be distinguished from it because one mutant that failed to localize, L27F, still bound adenylyl cyclase normally and maintained a wild type cAMP signaling system. Previous studies demonstrated that a proline-rich SH3-binding site, P2, is required for protein readily coprecipitated the untagged protein, which is seen as a 70-kDa band in Fig. 7. However, each of the three point mutants, L16P, R19T, and L27F, precipitated much less untagged protein. Because none of these proteins localized correctly, multimerization may be essential for directing CAP to actin cortical patches.

The experiments described so far have not directly addressed the role of actin in cAMP signaling but just the actin interacting regions of CAP. Thus, if actin were required for cAMP signaling through CAP-independent mechanisms we would have not detected it. Actin is encoded by Act1, which is an essential gene, so its role cannot be tested through knockout technology. Recently, however, the drug latrunculin-A was found to rapidly disassemble the yeast cytoskeleton by depolymerizing actin. In vitro, latrunculin-A binds actin monomers and prevents them from assembling into filaments (46). In vivo, the effects are rapid but are readily reversed when the drug is washed away. Thus, cells can be tested in the presence of the drug, and the drug can then be washed away to allow cells to grow. To determine whether the actin cytoskeleton mediated Ras interaction with adenylyl cyclase, we incubated wild type and Ras2Val19 cells with latrunculin-A, performed heat shock experiments, and then determined whether the Ras2Val19 cells became resistant to heat shock when they were treated with latrunculin-A (Fig. 8). We found no differences in viability upon drug treatment. That is, Ras2Val19 cells were still heat shock-sensitive when tested in the presence of latrunculin-A. This suggests that F-actin itself is not required for Ras to activate adenylyl cyclase.

We present evidence that the N terminus of CAP/Srv2p is required for localization to actin cortical patches. Three mutations within the extreme N terminus produced proteins that failed to localize to actin cortical patches as determined by immunofluorescence and GFP fusion tagging. This domain overlapped with the adenylyl cyclase-binding site but could be distinguished from it because one mutant that failed to localize, L27F, still bound adenylyl cyclase normally and maintained a wild type cAMP signaling system. Previous studies demonstrated that a proline-rich SH3-binding site, P2, is required for protein readily coprecipitated the untagged protein, which is seen as a 70-kDa band in Fig. 7. However, each of the three point mutants, L16P, R19T, and L27F, precipitated much less untagged protein. Because none of these proteins localized correctly, multimerization may be essential for directing CAP to actin cortical patches.

The experiments described so far have not directly addressed the role of actin in cAMP signaling but just the actin interacting regions of CAP. Thus, if actin were required for cAMP signaling through CAP-independent mechanisms we would have not detected it. Actin is encoded by Act1, which is an essential gene, so its role cannot be tested through knockout technology. Recently, however, the drug latrunculin-A was found to rapidly disassemble the yeast cytoskeleton by depolymerizing actin. In vitro, latrunculin-A binds actin monomers and prevents them from assembling into filaments (46). In vivo, the effects are rapid but are readily reversed when the drug is washed away. Thus, cells can be tested in the presence of the drug, and the drug can then be washed away to allow cells to grow. To determine whether the actin cytoskeleton mediated Ras interaction with adenylyl cyclase, we incubated wild type and Ras2Val19 cells with latrunculin-A, performed heat shock experiments, and then determined whether the Ras2Val19 cells became resistant to heat shock when they were treated with latrunculin-A (Fig. 8). We found no differences in viability upon drug treatment. That is, Ras2Val19 cells were still heat shock-sensitive when tested in the presence of latrunculin-A. This suggests that F-actin itself is not required for Ras to activate adenylyl cyclase.
proper subcellular localization. Consistent with the key role of this interaction in localization, SH3 binding is reduced in all of the N-terminal mutants. This result was unexpected because the SH3-binding site is about 350 amino acids away, and SH3-binding sites are generally a continuous sequence of amino acids. The P2 site is also likely to be continuous because we have found that this region is sufficient to confer SH3 binding and a 23-amino acid peptide derived from the region competes away Abp1-SH3 binding (28). The simplest explanation for our data is that mutations in the N terminus prevent access to the SH3-binding site. The mechanism that controls access to this SH3-binding site may require the formation of a multimeric complex because all of the mutants that failed to localize were also reduced in CAP-CAP association. A model that incorporates these observations is presented in Fig. 9. We propose that as a monomer, CAP efficiently binds actin and adenylyl cyclase but not Abp1-SH3. Upon dimerization, the SH3-binding site is exposed to direct CAP to the actin cytoskeleton.

In theory, CAP could serve as an adapter protein to direct adenylyl cyclase to actin cortical patches. For example, in mammalian cells, Ras activates Raf, in part by translocating it from the cytosol to the plasma membrane to direct it to protein kinases (3). However, we have documented three interactions...
between CAP and actin. They are direct binding to actin monomers through the C terminus, P2 SH3 domain targeting to cortical actin patches, and, as shown here, N-terminal targeting to actin cortical patches. We have mutated each binding site and made most combinations of double mutations without attenuating cAMP signaling. Finally, complete disruption of the actin cytoskeleton by recruiting actin through CAP. 100-fold by Ras-GTP (39). Thus, Ras may stimulate the assembly of the actin cytoskeleton by regulating SH3 binding to adenylyl cyclase activity is stimulated 10–20-fold by Ras-GTP (39). Thus, Ras may stimulate the assembly of the actin cytoskeleton by recruiting actin through CAP. Furthermore, we provide evidence for a novel cytoskeletal regulatory domain binding to adenylyl cyclase association but not interaction between Ras and the actin cytoskeleton. However, our data suggest that Ras regulation and actin regulation by CAP are independent functions. This does not necessarily rule out the possibility that Ras regulates actin through CAP. Furthermore, we provide evidence for a novel cytoskeletal regulatory domain in the N terminus of CAP. Because this is the region of CAP required for Ras regulation of actin assembly, Ras may regulate actin by modulating access to the SH3-binding domain through the N terminus. Consistent with this possibility, SH3 domain binding to actin assembly activity is stimulated 10–100-fold by Ras-GTP (39). Thus, Ras may regulate the assembly of the actin cytoskeleton by recruiting actin through CAP.

Acknowledgments—We thank Dr. Michael Wigler and Kathy O’Neill for discussing unpublished information, Tim Stearns and Roger Tsien for providing assistance with microscopy use, members of the lab for helpful discussions, and Amanda Schifl for critical reading of this manuscript.

References

1. Boguski, M. S., and McCormick, F. (1993) Nature 366, 643–654
2. Lowy, D. R., and Willumsen, B. R. (1993) Annu. Rev. Biochem. 62, 851–891
3. Marshall, M. S. (1995) FASEB J. 9, 1311–1318
4. Marshall, C. J. (1995) Cell 80, 179–185
5. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
6. Bar-Sagi, D., and Feramisco, J. R. (1986) Science 233, 1061–1068
7. Hall, A. (1998) Science 279, 509–514
8. Chang, E., Barr, M., Wang, Y., Jung, V., Xu, H.-P., and Wigler, M. (1994) Cell 79, 131–141
9. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985) Cell 40, 27–36
10. Gibbs, J. B., and Marshall, M. S. (1989) Microbiol. Rev. 53, 171–185
11. Broach, J. R. (1993) Curr. Opin. Genet. Dev. 1, 370–377
12. Field, J., Xu, H. P., Michael, T., Ballester, R., Sass, P., Wigler, M., and Colicelli, J. (1990) Science 247, 464–467
13. Minato, T., Wang, J., Akasaka, K., Okada, T., Suzuki, N., and Kataoka, T. (1994) J. Biol. Chem. 269, 20845–20851
14. Field, J., Vojtek, A., Ballester, R., Bolger, G., Colicelli, J., Ferguson, K., Gerst, J., Kataoka, T., Michael, T., Powers, S., Higgy, M., Rodgers, L., Wieland, L., Wheland, B., and Wigler, M. (1990) Cell 61, 319–327
15. Fedor-Chaiken, M., Deschentes, R. J., and Broach, J. R. (1996) Cell 82, 329–340
16. Mintaer, K. A., and Field, J. (1994) Cell Signalling 6, 681–694
17. Nishida, Y., Shima, F., Sen, H., Tanaka, Y., Yanagihara, C., Yamawaki-Kataoka, Y., Kariya, K., and Kataoka, T. (1998) J. Biol. Chem. 273, 28019–28024
18. Shima, F., Yamawoki-Kataoka, Y., Yanagihara, C., Tamada, M., Okada, T., Kariya, K.-I., and Kataoka, T. (1997) Mol. Cell. Biol. 17, 1057–1064
19. Field, J., Nikiwa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, B. A., and Wigler, M. (1988) Mol. Cell. Biol. 8, 2159–2165
20. Gerst, J. E., Ferguson, K., Vojtek, A., Wigler, M., and Field, J. (1991) Mol. Biol. Cell 11, 1248–1257
21. Vojtek, A., Haarer, B., Field, J., Gerst, J., Pollard, T. D., Brown, S., and Wigler, M. (1991) Cell 66, 497–505
22. Haarer, B. K., Petzold, A. S., and Brown, S. S. (1993) Mol. Cell. Biol. 13, 7864–7873
23. Freeman, N. L., Chen, Z., Horestein, J., Weber, A., and Field, J. (1995) J. Biol. Chem. 270, 5650–5659
24. Gieselmann, R., and Mann, K. (1992) FEBS Lett. 298, 149–153
25. Gottwald, U., Brokamp, R., Karakassoglou, I., Schleicher, M., and Noegel, A. A. (1996) Mol. Biol. Cell 7, 261–272
26. Hubberstey, A., Yu, G., Loewith, R., Lautka, C., and Young, D. (1996) J. Cell. Biochem. 60, 459–466
27. Zelazko, A., Protopenov, V., David, D., Lin, X., Lustgarten, V., and Gerst, J. E. (1996) J. Biol. Chem. 271, 18243–18252
28. Freeman, N. L., Lila, T., Mintaer, K. A., Chen, Z., Pahk, A. J., Ren, R., Drubin, D. G., and Field, J. (1996) Mol. Cell. Biol. 16, 548–556
29. Lila, T., and Drubin, D. (1996) Mol. Cell. Biol. 6, 367–385
30. Wang, J., Suzuki, N., Nishida, Y., and Kataoka, T. (1996) Mol. Cell. Biol. 16, 4087–4097
31. Richardson, L. C., Gibb, J. B., Marshall, M. S., Sigal, I. S., and Tatchell, K. (1987) Science 235, 1218–1221
32. Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S., and Wigler, M. (1987) Cell 48, 789–799
33. Camonis, J. H., Kalekin, M., Gendre, B., Garreau, H., Boy-Marcotte, E., and Jacquet, M. (1986) EMBO J. 5, 375–380
34. Jones, S., Vignais, M.-L., and Broach, J. R. (1991) Mol. Cell. Biol. 11, 2841–2846
35. Daniel, J., Becker, J. M., Enari, E., and Levitzki, A. (1987) Mol. Cell. Biol. 7, 3857–3861
36. Deng, W. P., and Nickoloff, J. A. (1992) Anal. Biochem. 200, 81–88
37. Heim, R., Cubitt, A. B., and Tsien, R. Y. (1995) Nature 373, 663–664
38. Mintaer, K. A., and Field, J. (1995) Methods Enzymol. 255, 468–476
39. Mintaer, K. A., and Field, J. (1999) Cell Signalling 11, 127–135
40. Gutierrez, C., and Fink, G. R. (eds) (1991) Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology. Vol. 194. Academic Press, Inc., New York
41. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) Cell 75, 791–803
42. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
43. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Science 263, 802–805
44. Sass, P., Field, J., Nikiwa, J., Toda, T., and Wigler, M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9303–9307
45. Fields, S., and Song, O. (1989) Nature 340, 245–246
46. Ayscough, K. R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D. G. (1997) J. Cell Biol. 137, 397–416
A Cytoskeletal Localizing Domain in the Cyclase-associated Protein, CAP/Srv2p, Regulates Access to a Distant SH3-binding Site

Jong Yu, Christopher Wang, Stephen J. Palmieri, Brian K. Haarer and Jeffrey Field

J. Biol. Chem. 1999, 274:19985-19991.
doi: 10.1074/jbc.274.28.19985

Access the most updated version of this article at http://www.jbc.org/content/274/28/19985

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 22 of which can be accessed free at http://www.jbc.org/content/274/28/19985.full.html#ref-list-1