A Conserved Proline-Rich Region of the *Saccharomyces cerevisiae* Cyclase-Associated Protein Binds SH3 Domains and Modulates Cytoskeletal Localization

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Received 7 February 1995/Returned for modification 2 May 1995/Accepted 8 November 1995

Saccharomyces cerevisiae cyclase-associated protein (CAP or Srv2p) is multifunctional. The N-terminal third of CAP binds to adenylyl cyclase and has been implicated in adenylyl cyclase activation *in vivo*. The widely conserved C-terminal domain of CAP binds to monomeric actin and serves an important cytoskeletal regulatory function *in vivo*. In addition, all CAP homologs contain a centrally located proline-rich region which has no previously identified function. Recently, SH3 (Src homology 3) domains were shown to bind to proline-rich regions of proteins. Here we report that the proline-rich region of CAP is recognized by the SH3 domains of several proteins, including the yeast actin-associated protein Abp1p. Immunolocalization experiments demonstrate that CAP colocalizes with cortical actin-containing structures *in vivo* and that a region of CAP containing the SH3 domain binding site is required for this localization. We also demonstrate that the SH3 domain of yeast Abp1p and that of the yeast RAS protein guanine nucleotide exchange factor Cdc25p complex with adenylyl cyclase *in vitro*. Interestingly, the binding of the Cdc25p SH3 domain is not mediated by CAP and therefore may involve direct binding to adenylyl cyclase or to an unidentified protein which complexes with adenylyl cyclase. We also found that CAP homologs from *Schizosaccharomyces pombe* and humans bind SH3 domains. The human protein binds most strongly to the SH3 domain from the *abl* proto-oncogene. These observations identify CAP as an SH3 domain-binding protein and suggest that CAP mediates interactions between SH3 domain proteins and monomeric actin.

In the yeast *Saccharomyces cerevisiae*, conversion of ATP to the second messenger cyclic AMP (cAMP) by adenylyl cyclase is activated by the G protein RAS (43) and the adenylyl cyclase-associated protein (CAP or Srv2p) (20). Regulation of RAS between its active GTP-bound state and its inactive GDP-bound state (11, 18) involves an interplay of two classes of proteins. Guanine nucleotide exchange factors (GEFs) catalyze GTP exchange onto RAS, thereby activating RAS, and GTPase-activating proteins accelerate the GTPase activity intrinsic to RAS, thereby inactivating RAS (5). In *S. cerevisiae*, RAS GTPase-activating proteins are encoded by *IRA1* and *IRA2* (42) and the RAS GEF is encoded by *CDC25* (7, 8, 37).

Regulation of cAMP levels by CAP is not well understood. *S. cerevisiae* CAP was first isolated as a 70-kDa component of the adenylyl cyclase complex (19). Genetic screens independently identified CAP as a positive regulator of adenylyl cyclase (16, 20). Although genetic experiments implicated CAP in RAS regulation of adenylyl cyclase *in vivo*, reconstitution experiments demonstrated that CAP is not required for RAS activation of adenylyl cyclase *in vitro* (32, 46). Studies of the phenotypes displayed by *cap* disruption strains identified a second function of CAP (20). CAP is important for organization of the actin cytoskeleton (26, 44), and recent studies suggest that this function is mediated through an actin monomer sequestering activity (22, 24). Mapping studies localized the cAMP signaling/adenylyl cyclase binding domain to amino ac-

ids 1 to 168 and the cytoskeletal regulatory domain to amino acids 368 to 526 (Fig. 1) (23). To date, a function for the middle third of CAP (amino acids 169 to 367), which contains a highly conserved proline-rich region, has not been identified (31, 45, 50).

Small proline-rich stretches of several proteins have recently been shown to bind Src homology 3 (SH3) domains (36). SH3 domains are small regions present in a large number of proteins that show similarity to regions of the *c-src* proto-oncogene protein. There have been over 60 SH3 domain-binding proteins (hereafter referred to as SH3 proteins) identified, including several in *S. cerevisiae*. Binding sites for several SH3 domains have recently been established (10, 35). For example, the consensus binding sequence for the *c-abl* SH3 domain is XPXXPPPΨXP (X = nonconserved residue, P = proline, Ψ = hydrophobic amino acid) (36). SH3 domains and their binding sites are often found in signaling proteins thought to mediate cytoskeletal interactions and also in proteins such as RAS GTPase-activating protein and SOS (RAS GEF) which interact with RAS (35). Because removal of SH3 domains results in activation of the oncogenic potential of the *src* and *abl* proto-oncogenes, SH3 domains may in some cases bind inhibitors of transformation (21, 28). A functional role for SH3 domain interactions has been established for only a few proteins. The SH3 domain of GRB2 recruits SOS to the cell membrane upon growth factor activation of receptors (14). SH3 domains are also present in several cytoskeleton-associated proteins and can direct reporter proteins to the actin cytoskeleton (3). This

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S. cerevisiae* CAP** **SH3 binding consensus sequences*abl:** XPXXPPPΨXP**src:** RXLPPLPRΨ

FIG. 1. Alignment of the proline-rich regions of various CAP proteins (single amino acid [aa] code). The diagram at the top depicts major functional regions of the *S. cerevisiae* CAP. P₁ and P₂ denote proline regions and also the *S. cerevisiae* peptides synthesized for competition assays (Table 2). *abl* and *src* SH3 binding consensus sequences are noted at the bottom (X = nonconserved residue, Ψ = hydrophobic amino acid).

finding has led to the suggestion that SH3 domains are involved in regulation of the cytoskeleton (9, 13, 35).

CAP is an attractive candidate for an SH3 signaling molecule because it is involved in RAS/cAMP signaling and cytoskeletal organization and it contains a region similar to SH3 binding domains of other proteins. We show here that SH3 domains bind CAP homologs from *S. cerevisiae*, *Schizosaccharomyces pombe*, and humans. We also demonstrate that the SH3 binding site maps to a region that is required for cytoskeletal localization of yeast CAP.

MATERIALS AND METHODS

Expression and purification of proteins. The SH3 domain probes were expressed as glutathione *S*-transferase (GST) fusion proteins by using plasmid vectors purchased from Pharmacia. The SH3 domain of Abp1p, amino acids 535 to 592, was cloned as a *Bam*HI fragment in pGEX3X to create pTDLSC (GST-Abp1p SH3) by using the 5' PCR primer GCC AAA GGG GAT CCC TTG GGC CAC AGC and the 3' primer GAC GCT GAC GGG ATC CTC TAG TTG CCC. pKM 13-13 expresses amino acids 1 to 134 of Cdc25p as a GST fusion protein (GST-Cdc25p SH3). This plasmid was constructed by using the 5' primer GGG GGA TCC ATG TCC GAT ACT AAC ACG and the 3' primer GGG GAA TTC GTG CTT TCT GAG ATG ACT GTC to amplify a fragment from yeast genomic DNA by using PCR. The amplified fragment was cloned into the *Bam*HI-*Eco*RI sites of the vector pGEX 4T-1. pNF453 encodes the two SH3 domains of Bem1p, amino acids 71 to 258 (GST-Bem1p SH3). This plasmid was constructed as described above by using the 5' primer NF200 (CTT AGA ATT CAC TTC TCC AGA GAA AGT TAT AAA A) and the 3' primer NF58 (CTT AAC CTC GAG AGA ACC AAG GCT AAT ATT ACT). The PCR-amplified fragment was cloned into the *Eco*RI-*Xho*I sites of the vector pGEX 4T-1. GST-Abp1p SH3 expresses amino acids 84 to 138 in pGEX 2-T (9). GST-Rvs167p encodes amino acids 428 to 477 of the Rvs167p SH3 domain (a generous gift from Pascal Durrens). GST fusion proteins were purified as described previously (41) and, when used for overlay assays, biotinylated as described previously (9).

An expression system for *S. cerevisiae* CAP expressed from the T7 promoter (pT7.CAP) has been described elsewhere (20). Expression plasmids for fragments of CAP were constructed by amplifying the appropriate regions by using PCR and cloning the fragments into plasmid pT7 to create plasmids pT7.CAPΔ29 (70C, GGA AAA AAG ACC ATA TGG ATT TTG C; 70B, ATT CAA AGT CGA CTT ATT TCT TTT A), pT7.CAPΔ30 (70A, GTG AAA TCG ACA TAT GCC TGA CT; 70D, GCA AAA TCG TCG ACT CAT TTT TTC CAG G), and pNFΔ17 (NF50, TCC TTA TAC ACA TAT GCT CGA GTC CTG

GGT TGC AGT GGA C; NF52, CTT GGA TCC CCT ACC AAT TCC TTT CTA GGA GGC CTC). Plasmid pNFΔ7 was made by deleting a 600-bp *Sau*I-*Eco*47III fragment from plasmid pT7.CAP. All T7 vectors were transformed into *Escherichia coli* BL-21(DE3) pLysS for protein expression. Plasmids expressing amino acids 337 to 376 (pTDL25 [5' primer GTC GCG GAT CCC TGA ATT ACG TCA ATC C; 3' primer GCG CGA ATT CCC TAC CAA TTC CTT TCT AGG]) and 176 to 337 (pTDL33 [5' primer GCG CGG ATC CTG TTC TCC TGG GTT GCA G; 3' primer GCG CGA ATT CGG ATT TTT GTG AGT TTG TTG]) of *S. cerevisiae* CAP were cloned as *Bam*HI-*Eco*RI fragments into pGEX3X and were expressed as GST fusion proteins. Other plasmids used were pTL, which expresses human CAP (31) as a hemagglutinin (HA) fusion protein (5' primer ACT CTG AGC AGG ATG TCG ACA ATG GCT GAC ATG CAA AAT CTG GTA; 3' primer TGT CAC TGT GGT TGT CGA CAT CTG TTA TCA CCA TAG GGT CTT GAA CTG), and pGST-spCAP1, which expresses *S. pombe* CAP1 as a GST fusion protein (both generous gifts from M. Kawamukai and M. Wigler). Human CAP was also expressed as GST fusion proteins. Appropriate DNA sequences were amplified from pTL by using PCR and cloned into *Bam*HI and *Xho*I sites of pGEX 4T-1. pNF249 encodes amino acids 1 to 461 (primers NF206 and NF54); pNF463 (primers NF222 and NF54) and pNF464 (primers NF206 and NF221) encode amino acids 284 to 461 and 1 to 247, respectively. pNF462 (primers NF220 and NF221) encodes the P₁ region, amino acids 213 to 247. The primers used for amplification were NF220 (CCT AGG ATC CGT GGC AAA AGA ACT GAG C), NF206 (TCC TTA TAC GGA TCC ATG GCT GAC ATG CAA AAT CTG), NF221 (CTT AAC CTC GAG TAA GCC TGA ACT GGT AGA GAC), NF222 (CCT AGG ATC CCC TGC CCT GAA GGC TCA G), and NF54 (CCT GGA TCC GGT TGT CGA CAT CTG TTA TCA CCA TAG GGT).

To determine the relative amounts of yeast and human CAP expressed by the yeast and bacterial expression plasmids, extracts were prepared and analyzed in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels stained with Coomassie blue. In addition, Western blots (immunoblots) were used to confirm expression of the various expression constructs by using either a polyclonal antiserum against yeast CAP (poly 155), monoclonal antibody (MAb) 12CA5, or an anti-GST polyclonal antiserum.

Peptides. The peptides P₁ (APAPPPPPPPAPPASV) and P₂ (YSKSGPPPPRPKKPSTLTKRPPR) were synthesized with a C-terminal NH₂ by the Protein Chemistry Laboratory of the Medical School of the University of Pennsylvania. The tyrosine residue was added to the P₂ peptide to provide an acceptor site for possible ¹²⁵I labeling. Poly-L-proline was purchased from Sigma Biochemicals (St. Louis, Mo.). Peptide concentrations were determined by weight, which yielded values within twofold of the concentration determined from the predicted extinction coefficient at A₂₆₀.

Coprecipitations. For coprecipitation experiments, the following reagents were mixed together in a 1.5-ml microfuge tube: ~10 μg of purified GST-SH3 probe, ~20 μg of purified CAP (or ~200 μg of an *E. coli* extract from a

TABLE 1. *S. cerevisiae* strains and plasmids used in this study

Strain	Genotype	Reference
SP1	<i>MATa leu2 his3 ura3 trp1 ade8 can1</i>	43
17-14D ^a	<i>MATa leu2 his3 ura3 trp1 ade8 can1 cap::HIS3 TRP LYS</i>	32
T18-5AT	<i>MATα his2 leu2 ura3 trp1 ade8 cyr1::URA3</i> (pYCA20)	32
T158-5H1	<i>MATa leu2 his3 ura3 trp1 ade8 can1 cyr1::URA3</i> (pYEP SCH9)	43
JFSKN34 ^a	<i>MATa leu2 his3 ura3 trp1 ade8 can1 cap::HIS3</i>	20
JF36A1	<i>MATα leu2 his3 ura3 trp1 ade8 can1 ras1::URA3 ras2::HIS3</i> (pEF-CYR1)	19
DDY817 ^a	<i>MATα his3Δ200 leu2-3,112 ura3-52 cap::HIS3</i>	This work

^a Disruption strain with a deletion of the *CAP* coding region between the *NsiI* and *HpaI* sites.

strain expressing CAP), ~10⁶ cpm of an extract prepared from a ³⁵S-labeled yeast culture (*cap* disruption strain JFSKN34 [Table 1]), and 20 μl of a 50% glutathione-Sepharose (Pharmacia) slurry. The mixture was brought to a final volume of 200 to 500 μl with buffer C [20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.2), 0.1 mM EGTA, 1.0 mM β-mercaptoethanol, 1.0 mM phenylmethylsulfonyl fluoride] and then incubated for 1 h at 4°C with gentle agitation. The beads were washed with buffer C containing 0.5 M NaCl and 1% Lubrol PX and then with buffer C alone. The beads were suspended and then boiled in SDS sample buffer (Bio-Rad, Richmond, Calif.), run on SDS-gels, and analyzed by fluorography.

Immunolocalization. We used modifications of published protocols to stain for CAP and actin in yeast cells (25). Yeast cells expressing various CAP deletion constructs have been previously described (23). Cells were grown to a density of ~5 × 10⁷/ml and fixed by addition of formaldehyde to a final concentration of 5% directly to the growth medium. Approximately 5 × 10⁶ to 5 × 10⁷ cells were washed with 1.2 M sorbitol–0.1 M potassium phosphate (pH 6.5)–1% β-mercaptoethanol, suspended in a volume of 1 ml of the same buffer, and then digested for 30 min at 37°C with 20 U of Zymolyase (ICN Biomedicals, Costa Mesa, Calif.). Digestion was monitored by phase-contrast microscopy. Cells were washed three times in 1.2 M sorbitol–0.1 M potassium phosphate (pH 6.5) and suspended in the same buffer. Coverslips, or multiwell slides, were coated with 0.1% polylysine solution and washed three times with distilled H₂O. Digested cells were transferred to coverslips and allowed to settle for 30 min. CAP was stained with an affinity-purified rabbit anti-CAP antibody, poly 155, that had been raised against CAP purified from an *E. coli* expression system. The antibody was affinity purified by chromatography against CAP cross-linked to CNBr-activated Sepharose beads (Pharmacia). A 1:10 dilution of the affinity-purified poly 155 was incubated for 1 h in blocking solution (1% bovine serum albumin and 0.05% Nonidet P-40 in phosphate-buffered saline [PBS] buffer) and then washed three times with PBS containing 0.05% Nonidet P-40 and three times with PBS alone at room temperature. Coverslips were incubated with blocking solution for 10 min. This was followed by a 1-h incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (Calbiochem, La Jolla, Calif.) diluted 1:200. Prior to use, the secondary immunoglobulin G was preincubated with a wild-type yeast strain (SP1) to absorb nonspecific yeast cell antibodies prior to the staining procedure. After the secondary antibody incubation, coverslips were washed as described above and then incubated for 1 h with a 1:200 dilution of phalloidin-tetramethylrhodamine B isothiocyanate (Fluka Chemical Corp., Ronkonkoma, N.Y.) to stain for actin. The coverslips were washed as described above and mounted with Gelvatol (Monsanto Polymers and Petrochemicals Co., St. Louis, Mo.) or mounting solution (0.1% phenylenediamine and 0.02% DAPI [4',6-diamidino-2-phenylindole dihydrochloride] in glycerol [pH 9.0]; both from Sigma). Slides were stored at –20°C protected from light. Cells were viewed with appropriate filters and photographed with TMax 400 film (Eastman Kodak, Rochester, N.Y.) by using either a Microphot-FXA microscope (Nikon, Melville, N.Y.) or an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence microscopy.

Other methods. Yeast and bacterial extracts were prepared as previously described (22). Affinity purification of *S. cerevisiae* CAP and various anti-CAP MAbs and polyclonal antisera have been described elsewhere (22). Protein concentration was determined by the method of Bradford (6), using bovine serum albumin as a standard, with reagents purchased from Bio-Rad. Adenylyl cyclase activity was measured as described previously (39). Growth and genetic manipulations of yeast strains were performed as described previously (25). The strains used in this study are described in Table 1. General molecular biology methods were carried out as described previously (40).

RESULTS

All CAP homologs contain a centrally located proline-rich region, designated P₁, that is similar to the consensus sequence for SH3 binding sites (Fig. 1). Most homologs also contain other potential SH3 binding sites nearby, such as the P₂ site

shown in Fig. 1. To determine if *S. cerevisiae* CAP is an SH3 protein, we expressed CAP in *E. coli* and then performed overlay experiments with probes derived from various SH3 domains. All SH3 domains were expressed and purified as GST fusion proteins. A biotin-based detection system in combination with either color development or chemiluminescence was used to visualize the binding signal. A representative blot probed with the SH3 domain from the yeast actin-binding protein Abp1p (13) is shown in Fig. 2A. A prominent band of 70 kDa was detected in the extract expressing yeast CAP (Fig. 2A, lane 8) and was also detected in a preparation of CAP purified by MAb affinity chromatography (Fig. 2A, lane 2). We also saw lower-molecular-weight bands that varied somewhat between constructs; some were evidently degradation products of CAP, as they were also recognized by a CAP antiserum (data not shown). Other proteins detected in these blots were *E. coli* proteins recognized by the detection system alone and were thus nonspecific. The control blot prepared by using biotinylated GST did not recognize CAP, although the nonspecific *E. coli* proteins were again recognized (Fig. 2B). We tested the SH3 domains of several other yeast genes and found binding to the P₂ region with probes from the SH3 domains of Rvs167p and Bem1p (data not shown). We also tested a probe that includes the SH3 domain from Cdc25p, the yeast RAS GEF (7, 8, 37). Although GST-Cdc25p SH3 probes did not recognize CAP on blots, GST-Cdc25p SH3 did bind the adenylyl cyclase complex (described below).

To localize the SH3 binding site in *S. cerevisiae* CAP, several deletion constructs expressing fragments of CAP were probed. Abp1p SH3 bound polypeptides expressed from plasmids pT7.CAP (full length), pT7.CAPΔ29, and pNFA17 but not pNFA7 and pT7.CAPΔ30 (Fig. 2A and C), despite the fact that all proteins were expressed at similar levels (not shown). These constructs allowed us to map the SH3 binding site between amino acids 248 and 367. This region contains the highly conserved P₁ sequence, amino acids 277 to 286 (PPPPPPAPPA), as well as the other potential site, P₂, amino acids 354 to 361 (PPPRPKKP). To distinguish which of these sites bound to Abp1p, we made two additional constructs which express amino acids 337 to 376 (pTDL25) and 176 to 337 (pTDL33) as GST fusion proteins. Only pTDL25 was recognized by Abp1p SH3, suggesting that the SH3 binding site localizes to the P₂ site (Fig. 2A, lanes 10 and 11).

As mentioned above, *S. cerevisiae* CAP also binds actin and adenylyl cyclase. To determine if CAP can associate with the adenylyl cyclase complex while bound to an SH3 domain, we performed coprecipitation experiments from yeast extracts with GST-SH3 domains and measured adenylyl cyclase activity precipitated by glutathione beads. A CAP polyclonal antiserum was used to monitor CAP association, and MAb 12CA5, which recognizes the HA epitope fused to adenylyl cyclase, was used to monitor the catalytic subunit. Tables 2 and 3 show that

FIG. 2. Abp1p SH3 binding to *S. cerevisiae* CAP. Total protein from *E. coli* extracts ($\sim 20 \mu\text{g}$) or, where indicated, affinity-purified recombinant yeast CAP ($\sim 1 \mu\text{g}$) was combined with SDS sample buffer and run on SDS-10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) and then probed with a biotinylated GST-Abp1p SH3 fusion protein (1.0 $\mu\text{g}/\text{ml}$) (A) or GST alone (B). Molecular sizes of prestained markers (Bio-Rad), in kilodaltons, are indicated. Binding was visualized by using streptavidin-horseradish peroxidase with enhanced chemiluminescence (A [lanes 1 to 8] and B) or color detection (A [lanes 9 to 12]). (C) Various regions of yeast CAP expressed in the constructs. Constructs pTDL25 and pTDL33 are expressed as GST fusion proteins (lanes 10 and 11). aa, amino acid.

GST-Cdc25p SH3 and GST-Abp1p SH3 both precipitated adenylyl cyclase activity from yeast extracts. GST-Abp1p SH3 failed to precipitate adenylyl cyclase from a *cap* disruption strain and also from an extract expressing a mutant adenylyl cyclase, pYCΔ20, which lacks the 66-amino-acid CAP binding site at the extreme C terminus (Table 1) (32). When peptides derived from the P₁ and P₂ sites were used as competitive inhibitors for adenylyl cyclase binding, the P₂ peptide was found to inhibit adenylyl cyclase precipitation by Abp1p SH3. The 50% inhibitory concentration was about 5 μM ; neither the P₁ peptide nor polyproline inhibited the precipitation of adenylyl cyclase at concentrations of up to 100 μM . This finding suggests that Abp1p SH3 binds the adenylyl cyclase complex through the P₂ site of CAP and is thus consistent with results of the overlay experiments shown in Fig. 2. Interestingly, Cdc25p SH3 precipitates adenylyl cyclase activity in all three strains that we tested, including the *cap* disruption strain and the strain expressing the mutant adenylyl cyclase (Table 2). Furthermore, neither P₁, P₂, nor polyproline inhibited Cdc25p SH3 binding (data not shown). Thus, Cdc25p SH3 may bind either adenylyl cyclase itself or an unknown adenylyl cyclase-binding protein.

To determine if CAP can associate with actin when bound to an SH3 domain, we performed coprecipitation experiments using ³⁵S-labeled extracts from a *cap* disruption strain. We have previously demonstrated that when recombinant CAP is added to these extracts, anti-CAP MAbs will coimmunoprecipitate a 46-kDa band that comigrates with yeast actin (Fig. 3, lane 7) (22). Similarly, the 46-kDa band was coprecipitated

with an SH3 probe in the presence of either full-length CAP or the C-terminal half of CAP (Fig. 3, lanes 4 and 5). CAP deletions missing the proline region failed to coprecipitate the 46-kDa band, which further supported the mapping data (data

TABLE 2. Precipitation of adenylyl cyclase activity in *S. cerevisiae* by various SH3 domains^a

Sample	cAMP (pmol/60 min)		
	JF36A1 (pEF-CYR1)	T158-5AT (pYCΔ20)	DDY817 (pEF-CYR1)
Protein A beads only	3	3	3
MAb 12CA5	385	127	769
CAP polyclonal antiserum 154	1,550	5	24
GST only	3	4	2
GST-Abp1p SH3	92	4	8
GST-Cdc25p SH3	52	62	458

^a A solubilized yeast extract was combined with 25 to 50 μg of GST-SH3 fusion protein and 20 μl of glutathione beads (50% solution). As indicated, antibodies against CAP or HA-tagged adenylyl cyclase were used as controls. After a 1-h incubation at 4°C, samples were washed with buffer C containing 0.2 M NaCl and adenylyl cyclase activity was measured as described previously (32). Yeast strains used: JF36A1(pEF-CYR1) (HA-tagged full-length adenylyl cyclase), T158-5AT (pYCΔ20) (HA-tagged 66-amino-acid C-terminal deletion of adenylyl cyclase), and DDY817(pEF-CYR1) (*cap* disruption strain). The data are the averages of duplicate datum points from a single experiment. Duplicate datum points usually vary between 0 to 20% in these assays. Adenylyl cyclase binding by the Abp1p SH3 probe and Cdc25p SH3 probe was observed in more than 10 independent experiments, though the relative binding by Cdc25p SH3, Abp1p SH3, and the two antibodies varied between experiments and preparations of extracts.

TABLE 3. Inhibition of GST-Abp1p SH3 binding to adenyl cyclase by proline rich peptides^a

Peptide concn (μ M)	cAMP (pmol/60 min)		
	Polyproline	P ₁	P ₂
0.00	100	100	100
0.01	130	71	86
0.1	103	111	87
1.0	107	108	66
10.0	116	107	4

^a An extract from strain JF36A1 was tested with GST-Abp1p SH3 as described for Table 2. Peptides P₁ and P₂ were derived from the *S. cerevisiae* CAP homolog. See Fig. 1 for peptide sequences.

not shown). Taken together, these observations suggest that *S. cerevisiae* CAP is an SH3 protein and that SH3 binding does not prevent CAP from binding either adenyl cyclase or actin.

The proline-rich domain is required to localize *S. cerevisiae* CAP to cortical actin patches. To examine the function of the proline-rich region of *S. cerevisiae* CAP, we determined the subcellular localization of CAP and several CAP deletion mutants via indirect immunofluorescence assays. An anti-CAP antiserum was purified by affinity chromatography and then used with an FITC-labeled secondary antiserum for localization. In wild-type budding cells, CAP localized to patches found primarily in the buds (Fig. 4A). There was also a fraction dispersed in the cytoplasm. As expected, no staining was detectable in *cap* disruption strains (Fig. 4C), and the patch staining was restored to *cap* cells by a CAP expression plasmid (Fig. 4E). Cortical patches, localized primarily in the buds of wild-type cells, are known to contain filamentous actin. Filaments can often be seen radiating from the buds to mother cells (30). To determine if CAP localized to cortical patches, we stained the same cells with the actin filament-binding agent phalloidin-tetramethylrhodamine B isothiocyanate. Figure 4B and F show that actin and CAP colocalize, suggesting that CAP is found in cortical actin patches.

To determine which regions of CAP are required for cortical

FIG. 3. Coprecipitation of yeast actin by SH3 domains in the presence of CAP. An extract was prepared from the *S. cerevisiae cap* disruption strain JF SKN34 labeled with ³⁵S. Where indicated, labeled extracts were incubated *S. cerevisiae cap* extracts (~20 μ g) and ~5.0 μ g of either GST alone or GST-Abp1p SH3. An anti-CAP MAb (JF2) was used as a control (lane 7). The samples were precipitated with glutathione beads (protein A was used with MAb JF2) and analyzed on an SDS-gel by autoradiography. The arrowhead indicates the migration position of yeast actin. GST alone and some GST fusion proteins also precipitate a background band of about 70 kDa (note that the band is not CAP because it was seen when *cap* disruption strains were used). F.L., full-length CAP. Deletion *S. cerevisiae* constructs are illustrated in Fig. 2. Sizes are indicated in kilodaltons.

FIG. 4. Localization of *S. cerevisiae* CAP. A polyclonal rabbit antiserum against CAP, poly 155, purified by chromatography against MAb affinity-purified yeast CAP, was used to localize CAP in *S. cerevisiae*. An FITC-conjugated anti-rabbit secondary antibody was used for CAP detection. Cells were also stained with phalloidin-tetramethylrhodamine B isothiocyanate to detect actin filaments. The strains and plasmids tested were wild-type strain SP1, *cap* disruption strain 17-14D, and 17-14D expressing full-length CAP. Similar localization results for wild-type cells were obtained when a MAb was used to detect CAP and an actin antibody was used to detect actin (data not shown).

patch localization, we performed immunolocalization experiments using an *S. cerevisiae cap* disruption strain harboring various CAP deletion plasmids (Fig. 5, diagrams). As expected, the full-length protein localized to cortical patches in the *cap* disruption strain (Fig. 4E and F). CAP C-terminal deletions (pADH-CAP Δ 8, pADH-CAP Δ 11, and pADH-CAP Δ 12) still colocalized to cortical patches (pADH-CAP Δ 11 data not shown). These deletions are deficient in the ability to correct the cytoskeletal defects of *cap* disruption strains, indicating a probable defect in actin binding. However, mutant proteins with the proline region deleted (pADH-CAP Δ 6, pADH-CAP Δ 7, pADH-CAP Δ 14, pADH-CAP Δ 15, and pADH-CAP Δ 16) did not localize to cortical patches but were found dispersed in the cytoplasm (pADH-CAP Δ 16; not shown). We noted that although pADH-CAP Δ 15 did not localize to cortical patches, this mutant localized to buds. Interestingly, in about 10 to 20% of the cells, mutants pADH-CAP Δ 6, pADH-CAP Δ 7, and pADH-CAP Δ 16 localized to ring structures. These rings were seen in either mother cells or nonbudded cells but not in daughter cells. Cells usually contained a single ring, but occasionally multiple rings were seen. In budding cells, there was no obvious correlation between the localization of a bud and the localization of the ring. Our antibodies failed to detect constructs with deletions in the N-terminal (adenyl cyclase binding) region of CAP in immunofluorescence experiments. Therefore, to address a potential role for adenyl cyclase in CAP localization, we carried out localization in a strain deficient in adenyl cyclase (Table 1). In this strain, we observed that CAP also colocalized with actin to cortical patches (data not shown). These experiments strongly suggest that neither adenyl cyclase binding nor actin binding is required to direct CAP to cortical patches. These data are consistent with a role for the SH3 binding site in determining CAP localization.

Conservation of the SH3 binding site. *S. cerevisiae* CAP is related to a family of actin-binding proteins. All family members have the centrally located major polyproline region (P₁),

FIG. 5. Localization of *S. cerevisiae* CAP mutants. Various deletion mutants of CAP were expressed in the *cap* disruption strain 17-14D and localized by using affinity-purified antiserum against CAP as described for Fig. 4. An illustration diagramming each deletion is adjacent to each micrograph. Similar results were obtained with each construct in at least five experiments using two independent transformants.

and several contain sequences similar to the *S. cerevisiae* SH3 binding site (P_2) (Fig. 1). To determine if the SH3 binding activity is conserved, we used several probes to test human and *S. pombe* homologs for SH3 binding. We were able to detect SH3 binding to the *S. pombe* homolog by using the *S. cerevisiae* Abp1p SH3 probe (Fig. 6). We also detected binding to the human homolog by the SH3 domain from the *abl* proto-oncogene (GST-*abl* SH3) (Fig. 7A). The *abl* SH3 binding site localizes between amino acids 213 and 247, which corresponds to the P_1 site. When the human homolog was combined with a ^{35}S -labeled yeast extract from a *cap* disruption strain, the GST-*abl* SH3 fusion protein coprecipitated a band that comigrated with yeast actin (Fig. 7B, lane 6), indicating that human CAP might bind simultaneously to actin and to SH3 domains.

DISCUSSION

We have demonstrated that the *S. cerevisiae* CAP binds the SH3 domains from several yeast proteins, including the actin-binding protein Abp1p. Binding by SH3 proteins was observed on blots probed with biotinylated SH3 probes and in coprecipitation experiments with adenyl cyclase and actin from yeast extracts. We have also been able to use Abp1p SH3 as an affinity ligand to purify recombinant CAP expressed in *E. coli* (22). The physiological importance of the SH3 binding site is supported by the observation that removal of this site leads to aberrant subcellular localization.

The SH3 binding site mapped to the proline-rich middle third of CAP. The SH3 domain from *abl* has been shown to bind to the consensus sequence XPXXPPPΨXP (X = noncon-

FIG. 6. Abp1p SH3 binding to *S. pombe*. A blot was prepared with *S. pombe* GST-CAP extract (~20 μ g of total protein) and probed with biotinylated GST-Abp1p SH3. Identical blots probed with biotinylated GST showed no binding to the CAP homologs (data not shown). Detection was by enhanced chemiluminescence using streptavidin-horseradish peroxidase (Amersham). The arrowhead indicates *S. pombe* GST-CAP. Sizes are indicated in kilodaltons. F.L., full-length CAP.

served residue, P = proline, Ψ = hydrophobic amino acid (36). There are critical prolines absolutely required for SH3 binding at residues 2, 7, and 10. Other SH3 domains contain critical prolines only at residues 7 and 10, thus leading to a consensus sequence of PXXP for all SH3 binding sites (10). Two sites within the middle third of *S. cerevisiae* CAP, P₁ (amino acids 275 to 286; PAPPPTPPAPPA [A = alanine]) and P₂ (amino acids 354 to 361; PPPRPKKP [R = arginine, K = lysine]), have this motif. In the *S. cerevisiae* homolog, one SH3 binding site is found between amino acids 337 and 367, strongly suggesting that the P₂ site is the primary, and perhaps only, SH3 binding site. The experiments with adenylyl cyclase allowed us to determine the 50% inhibitory concentration, which represents an upper limit for the K_D of Abp1p SH3 for the P₂ peptide. This value, 5 μ M, is similar to the highest reported affinities for SH3 peptide interactions (49). Although human CAP also binds SH3 domains, primarily that of *abl*, the binding region localized to the P₁ site amino acids 213 to 247. The *S. pombe* homolog contains a PXXP consensus at the P₂ site but lacks the PXXP consensus motif at the P₁ site, containing instead only a nine-amino-acid-long polyproline stretch. Thus, although all CAP homologs tested bind SH3 domains, the SH3 binding site localizes to different regions in the various homologs.

We also demonstrate that CAP colocalizes with actin to cortical patches and that without the P₂ domain, CAP is dispersed in the cytoplasm and is sometimes found in ring-like structures (Fig. 7). The localization region is best defined by the difference between $\Delta 8$ (missing amino acids 368 to 500), which localizes like the wild-type protein, and $\Delta 14$ (missing amino acids 356 to 526), which localizes aberrantly. $\Delta 8$ maintains the P₁ and P₂ regions, while $\Delta 14$ (and also the aberrantly localized $\Delta 15$ construct) is missing the P₂ region while maintaining the P₁ site. Therefore, of the three known protein-protein interactions involving CAP, adenylyl cyclase binding, actin monomer binding, and SH3 binding, only SH3 binding correlates with the observed localization. This observation suggests that SH3 proteins in the cortical patches modulate the cytoskeletal localization of CAP. SH3 domains and SH3 binding sites have been found to localize proteins to the cytoskeleton in mammalian cells (3, 38). Here, we demonstrate that an

SH3 binding site may also be a cytoskeletal targeting signal in yeast cells. The localization of the proline domain mutants of CAP to the ring-like structures and the localization of construct $\Delta 15$ to buds suggest that other sequences directing localization are found in the amino third of CAP. However, the physiological significance of these localizations is uncertain since they are not observed with full-length CAP.

Abp1p, a 65-kDa actin filament-binding protein, is an attractive candidate for an SH3 protein that binds CAP in cells because it also localizes to cortical actin patches (12). A number of synthetic lethal interactions between *ABP1* and other cytoskeletal genes have been found (1, 27). Two mutations are synthetically lethal with each other when each mutation by itself is viable, but the two mutations together are lethal. This is thought to reflect a functional redundancy of the protein products of the two genes. In these studies, *abp1* strains were found to be synthetically lethal with null mutations in *sac6*, *sla1*, and *sla2*. Other SH3 proteins that are candidates for in vivo targets of CAP include Rvs167p, which influences cytoskeletal and nutritional signaling (4), and Bem1p, which helps direct bud emergence. The SH3 domain of Rvs167p was recently reported to interact with actin in yeast two-hybrid assays (2). It is conceivable that the interaction with actin is indirect and mediated by the SH3 binding and actin binding domains of CAP. Experiments are in progress to determine if mutations in these candidate genes affect CAP localization.

We also found that a small fragment containing the SH3 domain from Cdc25p binds the CAP-cyclase complex. As the

FIG. 7. Abl SH3 binding to human CAP. (A) Overlay experiment. A blot was prepared and probed with a GST-*abl* SH3 fusion probe as described for Fig. 2 (note that a control blot prepared by using biotinylated GST showed no binding to human CAP [Fig. 2B and data not shown]). (B) Coprecipitation experiment with 35 S-labeled *S. cerevisiae*. The GST-*abl* SH3 domain was used to precipitate proteins from an extract prepared from 35 S-labeled *S. cerevisiae* (*cap* disruption strain JFSKN34). Where indicated, recombinant human CAP or *S. cerevisiae* CAP (precipitated with MAB JF2) was included. The experiment was performed as described for Fig. 3. The arrowhead indicates the migration position of *S. cerevisiae* actin. Sizes are indicated in kilodaltons. (C) Various human CAP deletions expressed as GST fusion proteins. The protein expressed from pNF249 does not migrate at its predicted size, probably as a result of degradation. aa, amino acid.

RAS GEF Cdc25p is an attractive candidate for an SH3 protein that interacts with the adenylyl cyclase complex in vivo. A direct binding to adenylyl cyclase is supported by the work in other laboratories. A mutation in *CDC25* has been shown to reduce the association of adenylyl cyclase with membrane fractions (34). In addition, most adenylyl cyclase activity was lost from the membrane fraction of *ras1 ras2 bcy1* strains (*bcy1* suppresses the normally lethal *ras1 ras2* double mutation because of a mutation in the regulatory subunit for the cAMP-dependent protein kinase) but was restored to the membranes when *CDC25* was overexpressed in these cells (15). Our data suggest that Cdc25p binds to the adenylyl cyclase complex in the absence of CAP because binding was observed in a *cap* disruption strain and a strain expressing a mutant adenylyl cyclase incapable of binding CAP. Thus, Cdc25p SH3 either binds directly to adenylyl cyclase itself or binds to another (as yet unidentified) component of the adenylyl cyclase complex.

CAP was isolated because of its physical association with adenylyl cyclase. It was also independently isolated in genetic screens as a chromosomal suppressor of the heat shock sensitivity of the hyperactive *RAS2^{Val-19}* allele (16, 20). Although the suppression of *RAS2^{Val-19}* implicates CAP in cAMP signaling in vivo, experiments in vitro found that CAP is not required for RAS to activate adenylyl cyclase (32, 47). Our data suggest that SH3 binding is important for subcellular localization of CAP, but the localization is not critical for cAMP signaling (23). This is because several deletion mutations that fail to localize to cortical patches still allow penetrance of the heat shock sensitivity phenotype in *cap RAS2^{Val-19}* strains. Interestingly, CAP binds very close to a RAS regulatory region found in the C terminus of adenylyl cyclase (32, 47). Thus, a direct interaction between RAS and CAP could still take place.

CAP is widely conserved in evolution, with homologs found in *S. cerevisiae*, *S. pombe* (29), *Caenorhabditis elegans* (8a), hydras (17), mice (45), rats (50), and pigs (24) and two in humans (31, 48). The porcine homolog, ASP-56, was originally identified as an actin-binding protein (24), and we recently demonstrated that the *S. cerevisiae* CAP also binds actin monomers (22). We now demonstrate the presence of SH3 binding sites in three CAP-related proteins. In *S. cerevisiae*, the SH3 binding domain is required to localize CAP to cortical patches, which are thought to be regions of the cell that are actively growing. Mulholland et al. have proposed that cortical patches help maintain the integrity of cells while the cell wall is disrupted during growth (33). In mammalian cells, CAP is enriched in the pseudopodia of migrating cells (45), also sites of change in the cell cytoskeleton. SH3 domains may also play a role in recruiting CAP to the actin cytoskeleton in mammalian cells. This would allow SH3 proteins to deliver actin monomers, bound by CAP, to sites of active actin polymerization and depolymerization. Consistent with this hypothesis, we have detected simultaneous binding of actin and SH3 domains to CAP homologs from both *S. cerevisiae* and humans (Fig. 3 and 7B). These studies should aid in the identification of signal transduction pathways that communicate with the cytoskeleton.

ACKNOWLEDGMENTS

We thank David Baltimore, Joseph Schlessinger, and Amita Sehgal for helpful discussions. We thank Ken Ferguson for constructing the T7 CAP expression plasmids $\Delta 28$, $\Delta 29$, and $\Delta 30$ and Kathy O'Neill for pTL. Additionally, we thank Jianhua Liu for technical assistance.

J.F. is supported by grants from the NIH and the Beckman Foundation. D.G.D. is supported by grants from the NIH and the American Cancer Society. The Protein Chemistry Laboratory of the Medical

School of the University of Pennsylvania is supported by core grants of the Diabetes and Cancer Centers (DK-19525 and CA-16520, respectively).

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