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Evidence for a Functional Link between Profilin and CAP in the Yeast S. cerevisiae

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Summary

CAP is a component of the S. cerevisiae adenylyl cyclase complex. The N-terminal domain is required for cellular RAS responsiveness. Loss of the C-terminal domain is associated with morphological and nutritional defects. Here we report that *cap*⁻ cells bud randomly and are defective in actin distribution. The morphological and nutritional defects associated with loss of the CAP C-terminal domain are suppressed by overexpression of *PFY*, the gene encoding profilin, an actin- and polyphosphoinositide-binding protein. The phenotype of cells lacking *PFY* resembles that of cells lacking the CAP C-terminal domain. Study of mutated yeast profilins and profilins from Acanthamoeba suggests that the ability of profilin to suppress *cap*⁻ cells is dependent upon a property other than, or in addition to, its ability to bind actin. This property may be its ability to bind polyphosphoinositides. We propose that CAP and profilin provide a link between growth signals and remodeling of the cellular cytoskeleton.

Introduction

RAS proteins form a class of low molecular weight guanine nucleotide–binding proteins that have been highly conserved in evolution and are thought to participate in signal transduction pathways that regulate growth and differentiation (Barbacid, 1987). Despite extensive studies, the identity of a RAS effector has been delineated in only one organism to date, the yeast Saccharomyces cerevisiae (Toda et al., 1985). In this yeast, RAS activates adenylyl cyclase activity in a complex that consists of at least two proteins: the adenylyl cyclase catalytic subunit, encoded by the *CYR1* gene; and an adenylyl cyclase–associated protein, encoded by the *CAP* gene (Field et al., 1990; Fedor-Chaiken et al., 1990).

Previous results indicate that CAP is a bifunctional signal transduction protein. The N-terminal domain of CAP, amino acids 1 to 168, appears to be both necessary and sufficient for full cellular responsiveness to mutant activated RAS proteins (Gerst et al., 1991). Disruption of the C-terminal domain of *CAP* in a wild-type RAS background is associated with nutritional and morphological defects: an inability to grow on rich medium (reflecting sensitivity to an excess of certain amino acids, particularly valine); sensitivity to nitrogen starvation; temperature sensitivity on minimal medium; and abnormally enlarged and round cells (Field et al., 1990; Gerst et al., 1991). These defects are complemented by overexpression of the C-terminal domain of CAP, amino acids 368 to 526 (Gerst et al., 1991), but are not suppressed by overexpression of positive or negative regulatory components of the RAS/adenylyl cyclase pathway, including cAMP phosphodiesterases, adenylyl cyclase itself, the regulatory or catalytic subunits of the cAMP-dependent kinase (Field et al., 1990), or the N-terminus of CAP (Gerst et al., 1991).

We have selected yeast genes on multicopy plasmids capable of suppressing the phenotypes that result from loss of the C-terminal functions of CAP. One of the genes we have found in our search is *PFY*, which encodes profilin (Magdolen et al., 1988).

Profilins have been characterized from a variety of sources, including human platelets, the protozoan Acanthamoeba, and the yeast S. cerevisiae. In all cases examined, profilins bind to, decrease ATP hydrolysis by, and inhibit the polymerization of actin monomers in vitro (Pollard and Cooper, 1986; Stossel et al., 1985; Haarer et al., 1990). Additionally, profilins from platelets and the protozoan Acanthamoeba bind polyphosphoinositides in vitro (Lassing and Lindberg, 1985, 1988; Machesky et al., 1990). When profilin is bound to polyphosphoinositides, it cannot bind actin monomers nor block actin polymerization (Lassing and Lindberg, 1985, 1988). These in vitro biochemical properties have led to the speculation that profilin is a component of a signal transduction cascade that modulates the cytoskeleton (Lassing and Lindberg, 1985, 1988). Other molecules within the cell, such as gelsolin, also bind actin and polyphosphoinositides (Janmey and Stossel, 1987) and are candidates for such regulatory molecules.

Disruption of the S. cerevisiae *PFY* gene provided the first genetic evidence that profilin function is required for cytoskeletal integrity. Cells lacking profilin are abnormally enlarged and round, do not contain actin cables, and bud randomly from the cell surface (Haarer et al., 1990). Similar defects in cell morphology and in actin distribution are seen in cells that overexpress the actin-binding protein ABP1 (Drubin et al., 1988) as well as in cells with aberrations in other known or suspected actin-binding proteins (Adams et al., 1989; Amatruda et al., 1990).

Profilins have also been reported to protect polyphosphoinositides from hydrolysis by phospholipase C-γ1 in vitro (Goldschmidt-Clermont et al., 1990; Machesky et al., 1990). This property correlates with the ability of profilins to bind polyphosphoinositides (Goldschmidt-Clermont et al., 1990; Machesky et al., 1990). In Acanthamoeba there are two profilins, isoform I and isoform II. Both bind actin with equal affinities, but isoform II binds polyphosphoinositides with 10- to 50-fold higher affinity than does isoform I,

Table 1. Strain Descriptions

Strain	Genotype	Reference
SP1	*MATa leu2 ura3 trp1 his3 ade8 can1*	CSHL collection
SKN32	*MATa leu2 ura3 trp1 ade8 can1 cap::HIS3*	Field et al., 1990
SKN37	*MATa leu2 ura3 trp1 ade8 can1 RAS2^{val19} cap::HIS3*	Field et al., 1990
PL1[a]	*ura3 trp1 his ade8 can1 pfy::LEU2*	This study
PU1[b]	*leu2 trp1 his ade8 can1 pfy::URA3*	This study

[a] Segregant from the diploid SP1/DC124 (CSHL collection) that had been transformed with the BamHI–HindIII fragment of pHBL1-1 to disrupt *PFY*.
[b] Segregant from the diploid SP1/DC124 that had been transformed with the BamHI–HindIII fragment of pU2-9 to disrupt *PFY*.

and commensurably protects polyphosphoinositides from hydrolysis by phospholipase C. These in vitro biochemical properties have led to the speculation that some profilins might participate in the generation of second messengers derived from the phosphatidylinositol cycle.

Further support for a role of profilin in phosphoinositide metabolism comes from more recent in vitro studies (Goldschmidt-Clermont et al., 1991). Growth factors such as platelet-derived growth factor and epidermal growth factor activate receptors that are tyrosine-specific protein kinases, and induce elevations of inositol phosphates (Berridge et al., 1984; Wahl and Carpenter, 1988; Wahl et al., 1989). Phospholipase C-γ1 also becomes tyrosine phosphorylated (Wahl et al., 1988, 1989; Meisenhelder et al., 1989; Margolis et al., 1989). This phosphorylation does not increase the activity of phospholipase C-γ1 toward polyphosphoinositides in artificial phospholipid bilayers, but it does render the polyphosphoinositides bound to profilin accessible to digestion (Goldschmidt-Clermont et al., 1991). One model suggested by this work is that the subcompartment of phosphoinositides bound to molecules like profilin is a source for second messengers, and is especially sensitive to growth signals that induce tyrosine phosphorylation of phospholipase C-γ1.

The yeast profilin gene on a multicopy plasmid suppresses both the nutritional and morphological defects of CAP-deficient cells. Cells lacking *PFY* have a phenotype that resembles in kind, though not in intensity, the phenotype of cells lacking *CAP*. Hence, we propose that CAP and profilin are components of the same signaling pathway. Our studies of mutant yeast profilins and the two profilin isoforms of Acanthamoeba have partially separated the functions of profilin: the ability to suppress the defects of *PFY*-deficient strains and the ability to suppress the loss of CAP function. Acanthamoeba profilin isoform II, the one with high affinity for polyphosphoinositides, can suppress more efficiently the defects of *CAP*-deficient strains than does isoform I, and hence we propose that CAP, in addition to its role in mediating the effects of activated RAS, may be a component of a polyphosphoinositide signal transduction pathway in S. cerevisiae.

Results

Isolation of the S. cerevisiae Profilin Gene

To isolate genes that when overexpressed suppress the sensitivity of *cap*$^-$ strains to growth on rich medium, we used the strain SKN37 (*cap::HIS3*; see Table 1), in which virtually the entire coding capacity of *CAP* has been deleted. SKN37 was transformed with a library of yeast genomic DNA in a 2μ high copy expression vector. Leu$^+$ transformants were scored for their ability to grow on rich medium (YPD) by replica plating. Out of approximately 4000 Leu$^+$ transformants analyzed, 12 exhibited a plasmid-dependent ability to grow on YPD. Plasmids were rescued from these transformants, and by Southern hybridization analysis, ten of those cross-hybridized. One of the ten was selected for further analysis. A restriction map of the smallest complementing region of this plasmid is shown in Figure 1A. The 1.8 kb region between the BamHI and HindIII sites was sequenced and found to contain the entire yeast *PFY* gene, encoding profilin (Magdolen et al., 1988). The plasmid was named pPFY. Deletion of the profilin open reading frame resulted in a plasmid unable to suppress the sensitivity of SKN37 to growth on rich medium (data not shown).

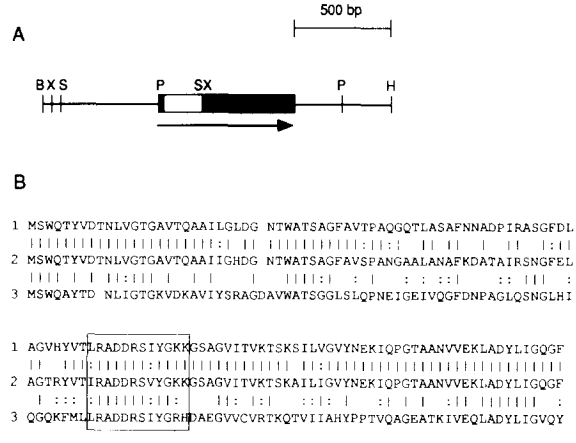

Figure 1. Restriction Map of the *PFY* Gene and a Comparison of Profilin Proteins

(A) Restriction map of the smallest complementing fragment of pPFY. Solid bars denote the location of the profilin open reading frame; open bar denotes the 208 bp intron. The arrow indicates the direction of transcription. Restriction enzymes are abbreviated as follows: B, BamHI; H, HindIII; S, SalI; P, PvuI; X, XhoI.

(B) A comparison of profilin proteins. The profilin proteins were aligned with the IntelliGenetics, Inc. GENALIGN program. 1, Acanthamoeba profilin isoform I (Pollard and Rimm, 1991). 2, Acanthamoeba profilin isoform II (Pollard and Rimm, 1991). 3, S. cerevisiae profilin. Vertical bars indicate identity; colons indicate similarity, using the Jimenez-Montano and Zamora-Cortina alphabet. Similar amino acids are grouped as follows: V, L, I, M; F, Y, W; K, R; E, D; Q, N; S, T; and A, G. Box indicates region analyzed by site-directed mutagenesis (see Table 3).

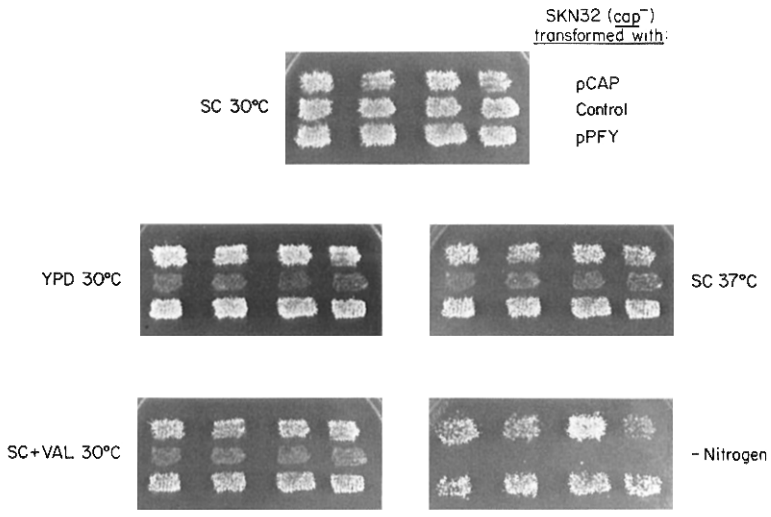

Figure 2. Suppression of *cap*⁻ Phenotypes by *PFY*

SKN32 was transformed with the indicated plasmids and plated onto SC – Leu plates. Independent transformants were patched onto SC – Leu plates, incubated 3–4 days at 30°C, and then replica plated to the following plates, which were incubated at the specified temperatures: SC – Leu at 30°C (SC 30°C); YPD at 30°C; SC – Leu with 6.5 mM valine at 30°C (SC + VAL 30°C); SC – Leu at 37°C (SC 37°C); and SC – Leu after a 7 day starvation on synthetic medium lacking a nitrogen source (– Nitrogen).

We next examined the ability of high copy plasmids expressing profilin to suppress the other defects in *CAP*-deficient strains. In the experiment shown in Figure 2, we transformed the strain SKN32 (*cap*::*HIS3*) with pPFY. pPFY suppressed these phenotypes of SKN32: inability to grow on YPD, sensitivity to valine, temperature sensitivity on synthetic medium, and sensitivity to nitrogen starvation. Thus overexpression of profilin suppresses the growth and nutritional defects associated with disruptions of the C-terminus of CAP. To explore the ability of profilin to complement the functions of the N-terminus of CAP, we transformed SKN37 with pPFY and subjected transformants to a heat shock. SKN37 contains the *RAS2*^{val19} allele, which renders *CAP*^{wt} strains heat shock sensitive. SKN37, because it lacks *CAP*, is heat shock resistant. Transformation of SKN37 with a plasmid expressing *CAP* restores heat shock sensitivity. The plasmid expressing profilin, by contrast, does not restore heat shock sensitivity to SKN37 (data not shown). These results indicate that profilin does not complement the N-terminal functions of CAP.

Organization of the Actin Cytoskeleton in CAP-Deficient Strains

CAP-deficient strains have an abnormal distribution of actin that can be corrected by overexpression of CAP or profilin (Figure 3). *cap*⁻ SKN32 cells transformed with the plasmid pCAP, which expresses the full-length CAP, exhibit the asymmetric actin distribution of a wild-type yeast cell (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Actin is present in thin filaments or cables in the mother cell, arrayed longitudinally along the mother-bud axis, and in cortical patches in the bud (Figure 3A). This asymmetric actin distribution is not observed in SKN32 cells transformed with the control plasmid YEp13M4 (Figure 3B). Rather, in these cells actin is present in cortical patches in both mother cell and bud, and actin cables are absent. In a small percentage of cells, actin bars are observed. Transformation of SKN32 with pPFY, which expresses profilin, restores an asymmetric actin distribution (Figure 3C): cables, although fainter than in wild-type cells, are again visible in the mother cells, and cortical patches are primarily, but not exclusively, localized to the bud.

Selection of Bud Sites in CAP-Deficient Strains

In the yeast S. cerevisiae, buds generally tend to emerge adjacent to previous bud sites. In haploid cells, the sites chosen are at one pole of the cell, whereas in diploid cells buds emerge from both poles (Sloat et al., 1981, and references cited therein). During budding, a ring of chitin is deposited at the neck of the bud; after cell division, the ring remains on the mother cell. Bud site selection can be readily assessed by staining these chitin-containing bud scars with the fluorescent dye Calcofluor. A ring of actin appears prior to chitin deposition and may play a role in bud site selection as well (Kilmartin and Adams, 1984).

CAP-deficient strains exhibit a random budding pattern that is corrected by overexpression of CAP and partially restored by overexpression of profilin (Figure 4 and Table 2). SKN32 cells transformed with pCAP primarily exhibit the wild-type haploid budding pattern: buds emerge adjacent to one another, as indicated by the clustered bud scars, and are at one pole of the cell (Figure 4A, left and center). Overexpression of CAP, however, leads to a 4-fold increase in bipolar budding, the typical pattern of **a**/α cells (Figure 4A, right; Table 1). This increase in bipolar budding in haploid cells has also been observed on overexpression of the actin-binding protein ABP1 (Drubin et al., 1988). *cap*⁻ SKN32 cells transformed with a control plasmid exhibit a random budding pattern (Figure 4B). Expression of profilin partially restores the polar budding pattern (Figure 4C).

Analysis of Profilin Gene Disruptions

Disruption of the profilin gene has been reported to lead to morphological defects that are strikingly similar to those we find in *cap*⁻ cells (Haarer et al., 1990). *pfy*⁻ yeast are abnormally large and round, and the actin distribution resembles that seen in *cap*⁻ yeast except that *pfy*⁻ cells usually contain actin bars. Both *pfy*⁻ and *cap*⁻ cells show increased chitin delocalization as cells increase in size (data

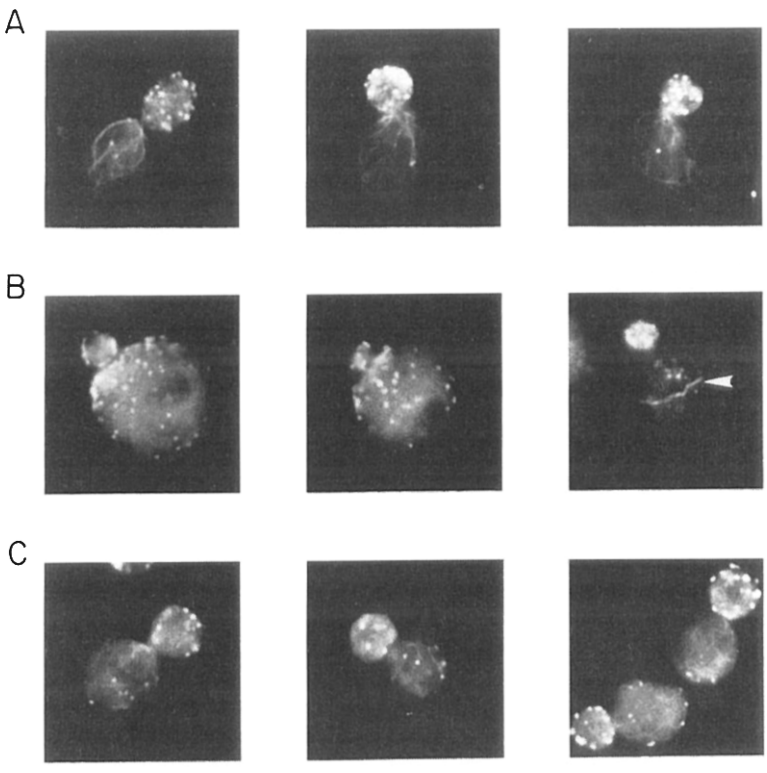

Figure 3. Effect of *CAP* or *PFY* on Actin Localization in *cap* Cells

SKN32 was transformed with pCAP (A), YEp13M4 (control) (B), or pPFY (C). Actin was visualized by indirect immunofluorescence. (The arrowhead denotes the location of an actin bar.)

not shown; Haarer et al., 1990). *pfy*⁻ cells also display a random pattern of bud scars, grow more slowly than wild-type cells, and are temperature sensitive for growth.

We have examined the growth characteristics of profilin disruptions in strains isogenic to our *cap*⁻ strains. One of the two copies of the profilin gene was disrupted in the diploid strain SP1/DC124 by integrating the 5.0 kb BamHI–HindIII fragment from pSK-HBL (Haarer et al., 1990) or

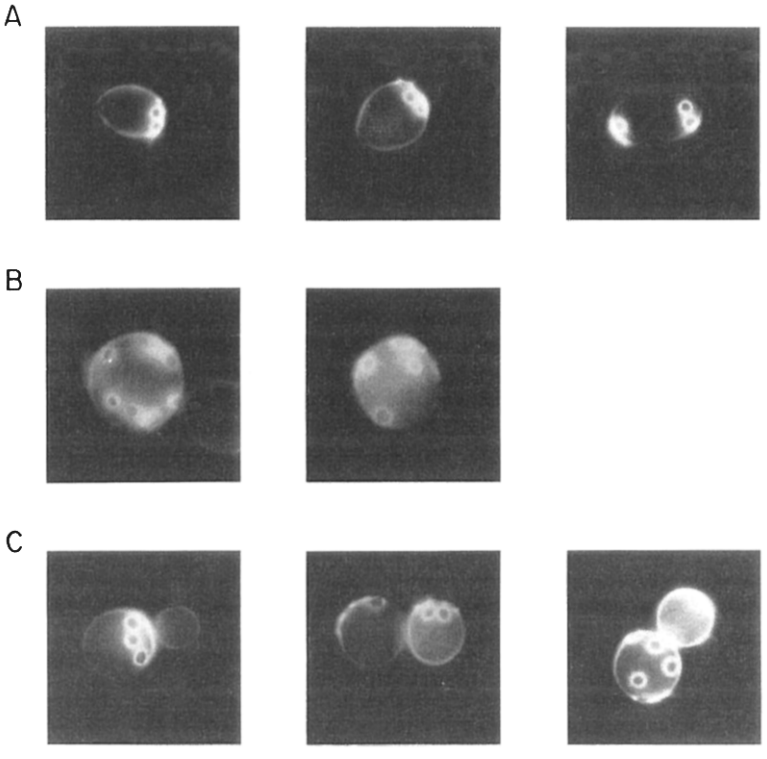

Figure 4. Effect of *CAP* or *PFY* on the Budding Pattern of *cap*⁻ Cells

SKN32 was transformed with pCAP (A), YEp13M4 (control) (B), or pPFY (C). Bud scars were visualized by staining with the fluorescent dye Calcofluor.

Table 2. Effect of Multicopy *CAP* or *PFY* on Bud Site Selection in a *cap*⁻ strain, SKN32.

Strain	Budding Pattern (%)		
	Polar	Bipolar	Random
SP1 (*CAP*^{wt})	92	4.5	3.5
SKN32 (*cap*⁻)	20	0.5	79.5
SKN32/pCAP	79	16	5
SKN32/pPFY	63.5	1.5	35

pCAP and pPFY are plasmids expressing full-length *CAP* and *PFY* genes, respectively. The budding pattern of various strains, grown in SC – Leu medium, was determined by staining cultures with Calcofluor and scoring at least 200 cells of each genotype with two or more bud scars.

Table 3. Location and Identity of Profilin Amino Acid Substitutions

Plasmid	Amino Acid Substitutions													*pfy*⁻	*cap*⁻
	71												82		
Wild type	L	R	A	D	D	R	S	I	Y	G	R	H		+	+
A111		E												+	–
A112						G								+	+
A115						G					K			+	–
A116						E								+	+
A119						G					G			+	–
A124		G									K			+	–

The sequence of the wild-type profilin is shown in the single-letter code. The plasmids contained wild-type or mutated *PFY* genes encoding the indicated amino acid substibutions. At right we indicate whether the designated plasmid was able to suppress (+) or failed to suppress (–) the phenotypic features of *pfy*⁻ or *cap*⁻ strains, PL1 and SKN32, respectively.

the 1.2 kb BamHI–HindIII fragment from pU2-9. In both pSK-HBL and pU2-9 the profilin-coding sequences have been replaced with auxotrophic markers, *LEU2* or *URA3*, respectively. Southern hybridization analysis confirmed that the fragments had integrated within one copy of the profilin gene. Diploid strains carrying these disruptions were subjected to tetrad analysis. Of the seven complete tetrads analyzed, the auxotrophic marker, *URA3* or *LEU2*, cosegregated with the known *pfy*⁻ phenotypes: round and enlarged cells, temperature sensitivity, and slow growth on minimal medium. In addition, *pfy*⁻ haploid cell growth was slightly retarded on YPD in comparison with synthetic medium. This last growth defect appears to reflect sensitivity to increased concentrations of certain amino acids such as valine, and is similar to, but not nearly as severe as, the growth defect of *cap*⁻ strains. When 6.5 mM valine is added to synthetic medium, growth of the *cap*⁻ strain SKN37 is totally inhibited, but the growth rate of the *pfy*⁻ strains is reduced only 30%–40% under these conditions (data not shown). The growth rate of wild-type strains is enhanced under these same conditions.

Characterization of Profilin Point Mutations

Yeast S. cerevisiae profilin most closely resembles the two profilins of Acanthamoeba (Figure 1B). These profilins contain several homology blocks, and the one between amino acid residues 71 and 82 of yeast profilin is particularly rich in positively charged amino acids. To distinguish those functions of profilin that are critical for suppression of a *cap*⁻ strain, we characterized a series of point mutations in arginine-72, arginine-76, and arginine-81 made in the S. cerevisiae profilin gene (Figure 5 and Table 3). Plasmids containing the point mutants of either or both arginine-72 and arginine-81 failed to suppress the phenotypes of the *cap*⁻ strain (Figure 5). In addition, *cap*⁻ cells transformed with these profilin point mutants grow slower on synthetic medium than do *cap*⁻ cells transformed with the control vector YEp13M4 (data not shown). Mutations in arginine-76 alone were not sufficient to cause loss of activity in this assay.

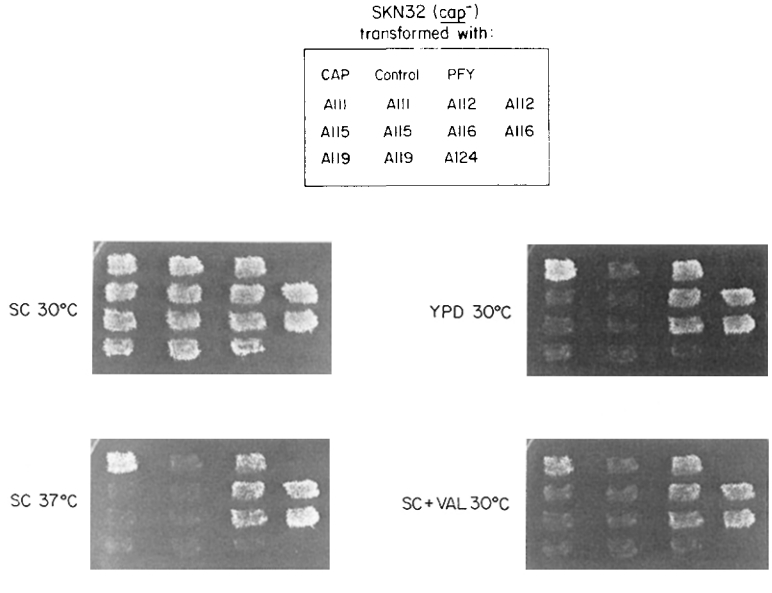

Figure 5. Effect of Profilin Mutants on *cap*⁻ Cells

SKN32 was transformed with the indicated plasmids (see Table 3 for mutant profilin sequences encoded by the A-series plasmids) and plated onto SC – Leu plates. Independent transformants were patched onto SC – Leu plates, incubated 3–4 days at 30°C, and then scored for growth on SC – Leu at 30°C (SC 30°C); SC – Leu at 37°C (SC 37°C); YPD at 30°C; and SC – Leu with 6.5 mM valine at 30°C (SC + VAL 30°C). The control plasmid is YEp13M4.

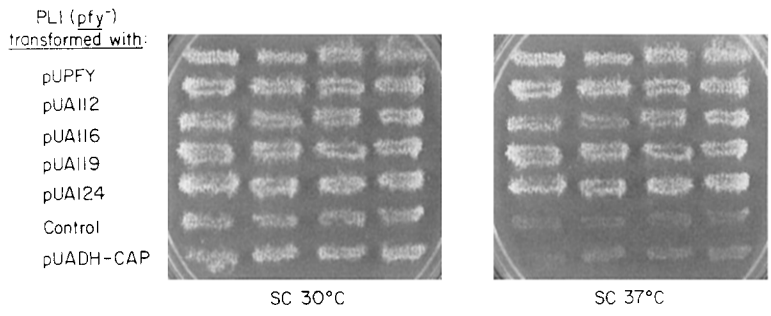

Figure 6. Suppression of a *pfy*⁻ Strain by Profilin Mutants

Strain PL1 was transformed with the indicated plasmids and plated onto SC – Ura plates. Independent transformants were patched onto SC – Ura plates, incubated 3–4 days at 30°C, and then replica plated to SC – Ura at 30°C (SC 30°C) and SC-Ura at 37°C (SC 37°C). The control plasmid is pUV2.

All the mutated profilins examined are comparable to wild-type profilin in their ability to suppress the slow growth and temperature sensitivity of the *pfy*⁻ strain (Figure 6). Moreover, all the point mutants bind to actin equally well when actin/profilin binding is assessed in vitro by Sepharose-bound DNAase I chromatography (B. H. and S. B., unpublished data). These results suggest that the ability of profilin to suppress the defects of a *cap*⁻ strain is dependent upon some additional function, perhaps one related to modulating a CAP-related signaling pathway.

Expression of Acanthamoeba Profilins in S. cerevisiae

The protozoan Acanthamoeba contains two profilins, isoform I and isoform II, that bind actin with equal affinities (Kaiser et al., 1986). The affinity of isoform II for polyphosphoinositides, such as phosphatidylinositol 4,5-bisphosphate, is 10- to 50-fold higher than that of isoform I (Machesky et al., 1990), and therefore we compared the two profilin isoforms for their abilities to suppress defects in *cap*⁻ strains. The Acanthamoeba profilins were expressed in yeast and tested by a replica-plating method for their ability to restore growth on rich medium to a *cap*⁻ strain. As shown in Figure 7, isoform II, but not isoform I, restores growth on rich medium to SKN37, although not as well as the wild-type yeast profilin. On prolonged incubation, strains transformed with isoform I grow weakly on rich medium. On the other hand, both isoform I and isoform II suppress the growth defects of a *pfy*⁻ strain (Figure 8). At higher temperatures, isoform I suppresses somewhat better than isoform II. In addition, isoform I appears to be present at higher levels than isoform II (B. H. and S. B., unpublished data). These results are consistent with the idea that suppression of a *cap*⁻ strain is dependent upon some additional function of profilin besides actin binding, and further suggest that this additional function may require an interaction with polyphosphoinositides.

Discussion

We have found that multicopy plasmids expressing profilin can suppress morphological and nutritional defects in cells lacking CAP. Only the functions due to loss of the C-terminus of CAP are corrected. The function of the N-terminus of CAP, namely, cellular responsiveness to the activated mutant RAS2^{val19}, is not restored by overexpression of profilin. The phenotypes of cells without profilin closely resemble the phenotypes of cells lacking the C-terminus of CAP (see Table 4): such cells are round and heterogeneously large, are often multinucleated, bud randomly from the cell surface, have aberrant actin distribution, are temperature sensitive, and show growth defects under certain nutritional conditions. Moreover, we have identified mutants of profilin that aggravate the CAP-deficient phenotype even in the presence of wild-type profilin. Altogether, these results indicate that CAP and profilin shape similar cellular events, and suggest that CAP and profilin operate on the same pathway.

CAP and profilin deficiencies both lead to randomized bud site selection. Several other yeast mutants with defects in budding have been previously described. These "bud" mutants can be classified into two groups: those

Table 4. Comparison of *cap*⁻ and *pfy*⁻ Yeast Strains

	Strain	
Phenotype	*cap*⁻	*pfy*⁻
Growth		
Synthetic medium	Slow	Slow
Rich medium	None	Reduced
Temperature sensitivity	Yes	Yes
Valine toxicity	Strong	Weak
Cell morphology	Enlarged, round, multinucleated	Enlarged, round, multinucleated
Actin distribution	No cables	No cables
	Cortical patches	Cortical patches
	Actin bars: rare	Actin bars: common
Budding pattern	Random	Random
Chitin distribution	Delocalized	Delocalized

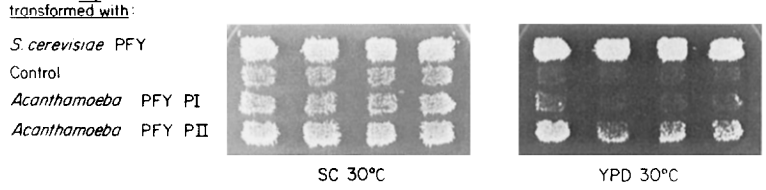

Figure 7. Effect of Acanthamoeba Profilins on *cap*⁻ Cells

SKN37 was transformed with the indicated plasmids and plated onto SC – Ura plates. Independent transformants were patched onto SC – Ura plates, incubated at 30°C, and then replica plated to SC – Ura (SC) and YPD at 30°C.

(*bud1–bud5*) that affect merely bud site selection (Chant and Herskowitz, 1991; Chant et al., 1991), and those (*cdc24*, *cdc42*, *cdc43*, and *cap2*) that all exhibit various aspects of the morphological defects seen in CAP- and profilin-deficient cells (Sloat et al., 1981; Adams et al., 1990; Amatruda et al., 1990). To date, none of the mutants of this latter group have been reported to display the sensitivities to growth conditions that typify CAP-deficient cells, nor have any of these genes been isolated as suppressors of CAP deficiencies in our screens of multicopy yeast genomic DNA. It will therefore be of interest to test these mutants for additional growth defects. Even though profilin mutants have only mild nutritional defects, the observation that overexpression of profilin can reverse the nutritional defects of CAP strains indicates that, like CAP, profilin can participate in two pathways: one affecting cytoskeletal function, the other affecting growth and viability under nutritional stress.

We have identified point mutants of profilin that seem to dissociate these two pathways. Profilins with amino acid substitutions at either arginine-72 or arginine-81 are not apparently altered in their ability to bind actin (B. H. and S. B., unpublished data), and their expression suppresses the loss of wild-type profilin, as shown here. However, expression of these mutant profilins does not suppress the loss of CAP. Thus, the ability of profilins to suppress loss of CAP may not be solely dependent on their cytoskeletal interactions, but may require an additional function, perhaps the ability to bind and modulate the hydrolysis of polyphosphoinositides.

Studies of the Acanthamoeba profilins support the idea that polyphosphoinositide binding is necessary for suppression of the CAP-deficient phenotypes. The two Acanthamoeba profilin isoforms, which differ at only a handful of residues, bind actin equally well (Kaiser et al., 1986), and we have shown that each is capable of suppressing

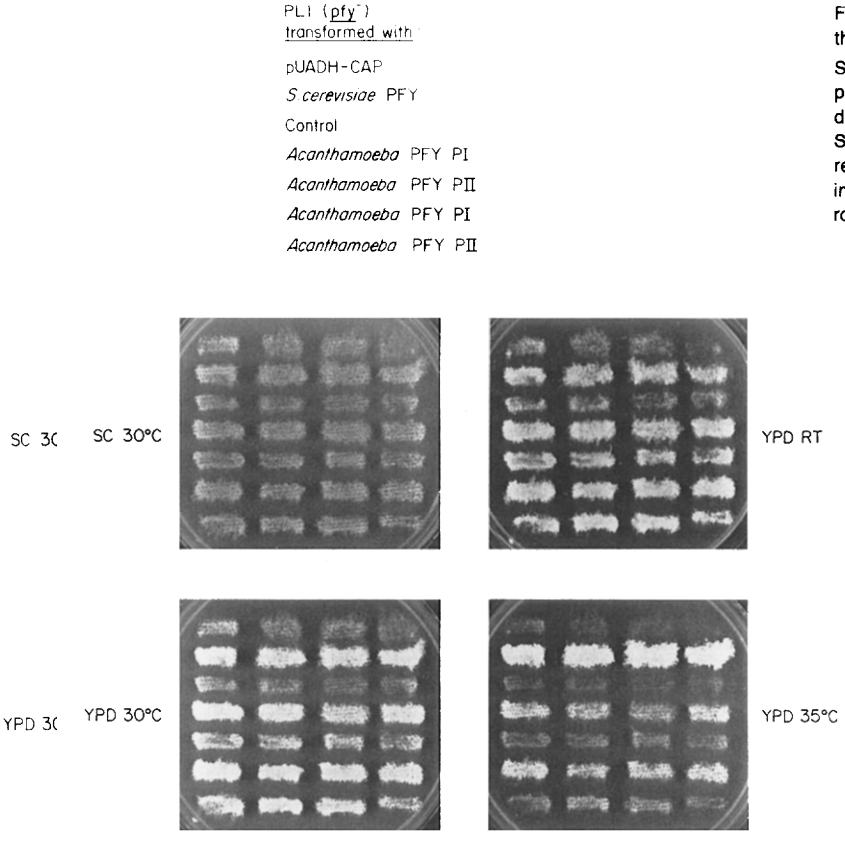

Figure 8. Suppression of *pfy*⁻ Strain by Acanthamoeba Profilins

Strain PL1 was transformed with the indicated plasmids and plated onto SC – Ura plates. Independent transformants were patched onto SC – Ura plates, incubated at 30°C, and then replica plated to SC – Ura (SC) and YPD and incubated at the specified temperatures. RT, room temperature.

the loss of wild-type profilin in S. cerevisiae. However, isoform II binds polyphosphoinositides with 10- to 50-fold greater affinity than does isoform I (Machesky et al., 1990) and is superior to isoform I in suppressing the growth defects of CAP-deficient cells.

These observations lead us to suggest that loss of CAP alters the formation of second messengers resulting from phosphoinositide metabolism, and that these second messengers are important in regulating cellular morphology and cellular adaptation to a variety of nutritional environments. Our data are consistent with a large number of specific models. Further biochemical and genetic analysis is required to resolve these models. Alterations in the metabolism of phosphoinositides might be apparent in mutants of either CAP or profilin, although significant changes in subcompartments of metabolites might not be measurable. If CAP affects phosphoinositide metabolism, then the expression of genes encoding phospholipases or phospholipid kinases might alter the CAP-deficient phenotype. It would be useful to characterize the precise biochemical defect of mutant profilins bearing substitutions at arginine-72 and arginine-81, or to demonstrate the existence of mutant profilins capable of suppressing loss of CAP function that cannot suppress loss of profilin function. Such studies are in progress.

Our experiments bring together multiple regulatory pathways in an unexpected fashion. CAP may somehow coordinate nutritional signaling with cytoskeletal rearrangements. We have no evidence that RAS controls CAP, but in yeast both RAS and CAP appear to interact with at least one common target, adenylyl cyclase. Although the relationship between RAS and adenylyl cyclase has not been maintained in mammalian cells, it is possible that a relationship between RAS, CAP, and profilin has been maintained in evolution. Identification of a mammalian *CAP* homolog or the heterologous expression of the S. cerevisiae *CAP* gene in mammalian cells may prove useful in establishing such a relationship.

Experimental Procedures

Strains, Media, and Genetic Manipulations

Yeast strains are described in Table 1. Yeast were grown on synthetic medium (SC; 0.67% yeast nitrogen base with ammonium sulfate, 3% glucose, and appropriate auxotrophic supplements) or on YPD (1% yeast extract, 2% Bacto-Peptone, 3% glucose) agar plates. Strains lacking *CAP* were grown on plates at 30°C, or in liquid cultures at room temperature; strains lacking profilin were grown at room temperature unless otherwise specified. Standard methods for yeast transformation, gene disruption, and tetrad dissection were employed (Rose et al., 1990). Replica plating methods were used to assay for sensitivity to growth on rich medium, temperature, heat shock, nitrogen starvation, and valine (Field et al., 1990; Gerst et al., 1991).

DNA Manipulations

Restriction endonucleases, ligases, and polymerases were purchased from New England Biolabs or Perkin-Elmer Cetus. Standard techniques and conditions were used (Maniatis et al., 1982). DNA was sequenced by the chain termination method using Sequenase version 2.0 according to the manufacturer's directions (United States Biochemical Corporation).

Plasmids

pPFY is a genomic profilin clone isolated from a library in the yeast vector YEp13M4 (J. Nikawa, unpublished data) by complementation of the sensitivity of SKN37 to growth on rich medium. pUPFY is a genomic profilin clone isolated from a library in the yeast vector pUV1 (J. Nikawa, unpublished data) by complementation of the YPD sensitivity of SKN37. Restriction map and Southern hybridization analysis indicated that pUPFY encoded profilin. pCAP is identical to pYEP-CAP, the genomic *CAP* clone described previously (Field et al., 1990). pUADH-CAP expresses the *CAP* gene under control of the *ADH1* promoter (Gerst et al., 1991).

Plasmid Constructions

The A-series profilin mutants were constructed by site-directed mutagenesis of profilin using the Bio-Rad (Richmond, CA) in vitro mutagenesis kit following the manufacturer's instructions. Single-stranded template DNA was produced from M13mp19 containing the entire profilin gene on a 1.8 kb BamHI–HindIII fragment; M13 was propagated in JM103. The following degenerate 49 base primer, corresponding to nucleotides 199 to 247 of the profilin-coding region, was used to introduce changes at basic residues: (A/G)AGTTCATGTTGTTG(A/G)(A/G)AGCTGACGAT(A/G)(A/G)AAGTATCTACGGT(A/G)(A/G)A(C/G)ATG. Altered profilin genes were identified by sequencing. In some cases, products of mutagenesis were screened by plaque hybridization using the following primer (corresponding to nucleotides 214 to 228 of the profilin coding region): AGAGCTGACGATAGA. Mutated profilin genes were isolated from the M13 vector as 1.8 kb BamHI–HindIII fragments and inserted into the BamHI–HindIII sites of YEp102 and YEp13M4. (YEp102 consists of the 2μ-containing 2.2 kb EcoRI fragment from YEp24 [Botstein et al., 1979] inserted into the EcoRI site of YIp5 [Botstein et al., 1979].)

The Acanthamoeba profilin fusion genes were generated as described below. Full-length cDNAs encoding Acanthamoeba profilin isoform I (PI) and isoform II (PII) were cloned as ~700 bp EcoRI fragments in pBluescript KS+ (Stratagene, La Jolla, CA) (Pollard and Rimm, 1991). The coding regions for these genes were placed next to the yeast profilin promoter as follows: First, a 500 bp SalI–PvuI fragment containing 5′ noncoding sequences from the yeast profilin gene was blunt-ended with mung bean nuclease (to generate a fragment spanning positions −508 to −11) and inserted into the blunt-ended (with Klenow) XbaI sites of the PII-containing plasmid (XbaI digestion removes all but 12 bases of the 5′ noncoding region of PII). The PvuI–XbaI junction of the resulting plasmid (PFY-PII) was confirmed by sequencing. Second, the corresponding yeast promoter–profilin I plasmid (PFY-PI) was generated by replacing the PII coding region, from a SalI site at codon 7 through the end of the cDNA, with the corresponding region from the PI clone. PI and PII cDNA sequences are identical from the initiator ATG through this SalI site. Third, the inserts of plasmids PFY-PI and PFY-PII were moved as EagI–XhoI fragments to the yeast vector YEp102 to generate plasmids YEp(PFY-PI) and YEp(PFY-PII).

The profilin gene disruption plasmid, pU2-9, was constructed by deleting the coding region of profilin, using the polymerase chain reaction (PCR), and replacing it with the *URA3* auxotrophic marker. The template for the PCR was pP1-7B2, which contains the profilin gene and 5′ and 3′ flanking sequences on a 1.8 kb BamHI–HindIII fragment in pUC119. The PCR utilized an oligonucleotide primer complementary to the upstream flanking sequence of profilin, 5′-CTAGGATATCCCAAGACATAATTTGC-3′, and one complementary to the downstream flanking region of profilin, 5′-TACTGATATCGTGTTCAATACTAAT-3′; both primers contain an EcoRV restriction endonuclease site. The 3′ ends of both primers were oriented outward from the profilin coding sequence so as to amplify a 4.5 kb region containing only the 5′ and 3′ flanking sequences of profilin (but not the profilin coding sequence) and the vector backbone. The PCR product was purified by gel electrophoresis, digested with EcoRV, and ligated to generate plasmid pP2. In plasmid pP2, the profilin coding sequence between nucleotides +9 and +340 was deleted and an EcoRV site inserted at the site of the deletion as a result of the PCR and subsequent steps. The Klenow-treated 1.1 kb HindIII *URA3* gene fragment was then inserted into the EcoRV site of pP2 to generate pU2-9.

Fluorescence Microscopy Methods

Actin was visualized with affinity-purified rabbit anti-actin primary antibody (Haarer et al., 1990) and FITC-conjugated goat anti-rabbit IgG secondary antibody (Sigma), as described (Pringle et al., 1989). Chitin was stained with the fluorescent dye Calcofluor (Pringle et al., 1989).

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