In Vivo Importance of Actin Nucleotide Exchange Catalyzed by Profilin

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Abstract. The actin monomer-binding protein, profilin, influences the dynamics of actin filaments in vitro by suppressing nucleation, enhancing nucleotide exchange on actin, and promoting barbed-end assembly. Profilin may also link signaling pathways to actin cytoskeleton organization by binding to the phosphoinositide PIP$_2$ and to polyproline stretches on several proteins. Although activities of profilin have been studied extensively in vitro, the significance of each of these activities in vivo needs to be tested. To study profilin function, we extensively mutagenized the *Saccharomyces cerevisiae* profilin gene (*PFY1*) and examined the consequences of specific point mutations on growth and actin organization. The actin-binding region of profilin was shown to be critical in vivo. *act1-157*, an actin mutant with an increased intrinsic rate of nucleotide exchange, suppressed defects in actin organization, cell growth, and fluid-phase endocytosis of *pfy1-4*, a profilin mutant defective in actin binding. In reactions containing actin, profilin, and cofilin, profilin was required for fast rates of actin filament turnover. However, Act1-157p circumvented the requirement for profilin. Based on the results of these studies, we conclude that in living cells profilin promotes rapid actin dynamics by regenerating ATP actin from ADP actin–cofilin generated during filament disassembly.

Key words: yeast • cytoskeleton • cofilin • filament turnover • ATP

Introduction

The ability of the actin cytoskeleton to undergo rapid rearrangements is critical for such cellular processes as cell polarity development, endocytosis, cell motility, and cytokinesis. Actin dynamics are modulated by a host of actin-binding proteins (Pollard, 1994). One such protein, profilin, binds to actin monomers and may, through its ability to interact with several proteins and phosphoinositides, link signaling pathways to the actin cytoskeleton (Machesky and Pollard, 1993). Profilin can impact actin dynamics by sequestering actin monomers (Carlsson et al., 1977), increasing the rate of exchange of ATP for ADP on actin (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991), and promoting barbed-end assembly of actin filaments (Pantaloni and Carlier, 1993; Kang et al., 1999). Profilin also increases the rate of actin filament turnover in a synergistic manner with cofilin, a protein that promotes disassembly of actin filaments at their pointed ends (Didry et al., 1998).

In all organisms studied to date, profilin plays a critical role in establishing and maintaining the proper organization of the actin cytoskeleton (Ayscough, 1998). We have chosen to study profilin function in the yeast *Saccharomyces cerevisiae* because it is a genetically tractable organism with a dynamic actin cytoskeleton. The yeast actin cytoskeleton consists of cables, which orient along the mother-bud axis, and cortical patches, which polarize in the developing bud and reorganize depending on the specific stage in the cell cycle (Pruyne and Bretscher, 2000). The importance of profilin in the organization of the yeast actin cytoskeleton has been demonstrated by analysis of yeast lacking profilin. These cells grow extremely slowly, become large and rounded, lack detectable actin cables, and no longer polarize their actin patches to the bud (Haarer et al., 1990). In addition to interacting with actin, yeast profilin (Pfy1p) binds to stretches of proline residues in the FH (formin homology) domains of Bni1p and Bnr1p (Kohno et al., 1996; Evangelista et al., 1997; Imamura et al., 1997). Members of the FH protein family bind to pro-

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Table I. Integrated Alleles of PFY1

Allele	Mutation	Feature	Growth		DDY strain number
			YPD*	YPD ± formamide[‡]	
PFY1	None	Wild-type	Wild-type	Wild-type	2044
pfy1-2	R75A	Charged	Wild-type	Wild-type	2011
pfy1-3	R90A, K92A	Charged	Wild-type	Wild-type	2030
pfy1-15	R23A, R75A	Charged	Wild-type	Wild-type	2027
pfy1-17	R23A, K92A	Charged	Wild-type	Wild-type	2029
pfy1-4	K66A	Actin binding	Conditional (25–34°C)	Lethal (37°C)	2008
pfy1-5	R71A	Actin binding	Wild-type	Lethal (37°C)	2014
pfy1-6	R80A	Actin binding	Wild-type	Lethal (37°C)	2025
pfy1-16	K66A, R80A	Actin binding	Conditional (25–34°C)	Not determined	2038
pfy1-7	H81A	Actin binding	Wild-type	Wild-type	2017
pfy1-8	D73A, D74A	Charged	Wild-type	Wild-type	2012
pfy1-9	D7A	Polyproline binding	Wild-type	Wild-type	2019
pfy1-10	Q3A	Polyproline binding	Wild-type	Wild-type	2020
pfy1-11	S1A	Polyproline binding	Wild-type	Wild-type	2023
pfy1-12	W29A	Polyproline binding	Lethal	Not determined	2003
pfy1-13	W2A	Polyproline binding	Conditional (25–34°C)	Not determined	2036
pfy1-14	Y119A, Y125A	Polyproline binding	Conditional (25–34°C)	Lethal (37°C)	2033
pfy1-18	Y119A	Polyproline binding	Wild-type	Lethal (37°C)	2041
pfy1Δ	Deletion	Profilin null	Conditional (25–30°C)	Lethal (37°C)	2034

*Indicated in parentheses is the permissive temperature range for growth on YPD. Temperatures tested were 16, 25, 30, 34, and 37°C.
[‡]Indicated in parentheses is the temperature at which growth of YPD + 2.5% formamide is lethal.

teins of the Rho family of small GTPases and are involved in cytokinesis and the establishment of cell polarity (Wasserman, 1998). The interaction between profilin and FH proteins thus provides a link between signaling by small GTPases and the actin cytoskeleton.

Although the interactions of profilin with actin and polyproline have been studied extensively biochemically, the significance of each of these activities in vivo has been much more difficult to test. The importance of the ability of profilin to enhance nucleotide exchange on actin has been controversial. Mammalian and amoeba profilins increase the rate of nucleotide exchange on actin in a concentration-dependent manner, by as much as 1,000-fold (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991; Vinson et al., 1998; Selden et al., 1999). However, plant profilins do not affect nucleotide exchange of rabbit muscle actin (Perelroizen et al., 1995, 1996), and yeast profilin only increases the rate of yeast actin nucleotide exchange approximately threefold (Eads et al., 1998). Furthermore, in *Xenopus* extracts, actin monomers are primarily associated with ATP rather than ADP (Rosenblatt et al., 1995), suggesting that nucleotide exchange might not be a rate-limiting step during actin assembly. However, profilin may be required to desequester and/or promote nucleotide exchange on actin bound by actin monomer-binding proteins, such as cofilin and thymosin β-4 (Pantaloni and Carlier, 1993; Blanchoin and Pollard, 1998). Data presented here indicate that the ability of profilin to enhance nucleotide exchange on actin is an important function in vivo.

Materials and Methods

Plasmid Construction and Mutagenesis

All mutations were generated using the QuikChange mutagenesis kit (Stratagene). The *PFY1* gene encoding yeast profilin was PCR amplified from genomic DNA 301 bp upstream and 159 bp downstream of *PFY1* using 5′ and 3′ primers containing engineered SacI and KpnI sites, respectively. The PCR product was cloned into pBluescript to generate pAW1. To mark *PFY1* with the *LEU2* auxotrophic marker, a NotI site was introduced into pAW1 93 bp upstream of the *PFY1* gene to generate pAW2. The *LEU2* fragment from pDD802 was ligated to NotI-digested pAW2 to generate pAW3.

All mutations in the PFY1 gene were generated using pAW2 as a template. After mutagenesis and verification of the mutation by restriction enzyme digestion and DNA sequencing, *LEU2* was subcloned into the NotI site of each mutated plasmid.

The plasmid for overexpression of yeast profilin in *Escherichia coli* was pMW172 (Eads et al., 1998). To overexpress mutant profilins, pMW172 was mutagenized.

Yeast Strain Construction

Standard techniques were used to grow, manipulate, and transform yeast strains (Rose et al., 1989). Complete disruption of the *PFY1* gene was performed using PCR-mediated gene replacement as described (Goode et al., 1998), using the *HIS3* plasmid pRS303 (Sikorski and Hieter, 1989). The disruption was generated in yeast strain DDY1102 to create strain DDY2001 (*MAT a/α ade2-1/+ his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 +/lys2-801 +/pfy1Δ::HIS3 ura3-52/ura3-52*). DDY2001 was sporulated to generate the *PFY1* deletion strain DDY2034 (*MAT a his3Δ200 leu2-3,112 lys2-801 pfy1Δ::HIS3 ura3-52*).

Plasmids carrying either *PFY1* or the mutant *pfy1* alleles marked with *LEU2* were digested with BssHII and transformed into DDY2001 for integration at the *pfy1Δ::HIS3* site. His⁻, Leu⁺ colonies were selected, and the presence of the mutation was verified by PCR and restriction enzyme digestion. Diploids were sporulated to generate haploid strains carrying *PFY1* or mutant *pfy1* alleles. The genotype of each strain was identical to that of the *pfy1Δ* strain DDY2034, except *pfy1* alleles were marked with *LEU2*. The DDY strain numbers for the strains used in this study are listed in Table I.

Phenotypic Analysis and Microscopy

For phenotypic analysis of each *pfy1* allele, strains derived from two independent transformants were examined. Growth on formamide was examined on YPD plates supplemented with 2.5% formamide at 25 and 37°C. Actin organization was examined by fixing and staining cells grown at 23°C, or shifted to 37°C for 4 h, with rhodamine-phalloidin (Cope et al., 1999). Uptake of Lucifer yellow by fluid phase endocytosis was performed
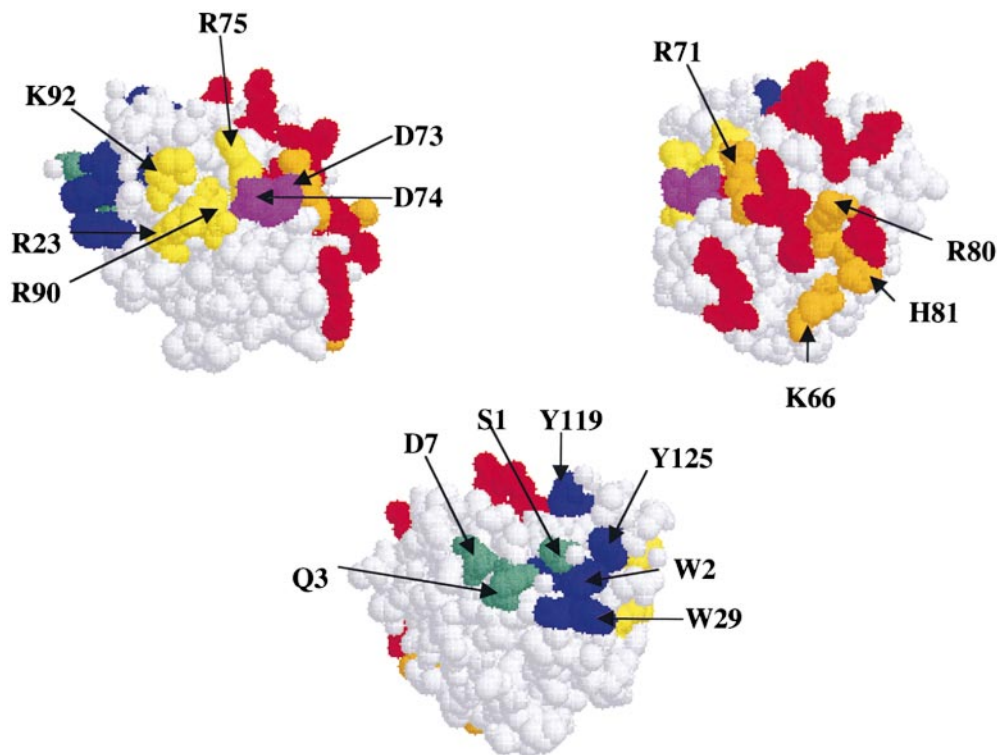
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Figure 1. Space filling model of yeast profilin, showing three rotations. Residues mutated in this study are indicated by arrows. Residues are color coded to illustrate function. Residues predicted to contact actin are red and orange. Orange residues were mutated to alanine. Residues forming the polyproline binding site are blue and green. Blue residues are hydrophobic and are predicted to have stronger effects on polyproline binding than green residues. Conserved basic residues and acidic residues are colored yellow and purple, respectively.

as described (Belmont et al., 1999b), except that the wash buffer was of 50 mM potassium phosphate, pH 7.5, 10 mM NaN₃, and 10 mM NaF.

Protein Purification

Recombinant yeast profilin was purified from *E. coli* as described for wild-type cofilin expressed as a nonfusion protein (Lappalainen et al., 1997), except that buffers contained 20 mM Tris, pH 8.0. Profilin concentration was determined spectrophotometrically as described (Eads et al., 1998).

Yeast cofilin was purified as a GST fusion protein and cleaved from GST by thrombin digestion (Lappalainen et al., 1997). Yeast actin was purified using DNAse I affinity chromatography as described (Rodal et al., 1999).

In Vitro Assays

Binding of profilin to monomeric actin was measured by determining the amount of actin that copelleted with profilin bound to poly-L-proline (PLP)[1] Sepharose. PLP–Sepharose was prepared as described (Rozycki et al., 1991). Variable concentrations of profilin were incubated with excess PLP–Sepharose beads in G-actin buffer (10 mM Tris, pH 7.5, 0.2 mM CaCl₂, 0.5 mM ATP, 0.2 mM DTT) at 4°C for 1 h. The beads were then washed three times with G-actin buffer, and G-actin was added to a final concentration of 5 μM. Samples were incubated at 4°C for 1 h. Beads were washed once with G-actin buffer, twice with G-actin buffer plus 100 mM NaCl, and were then resuspended in SDS sample buffer and subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue and quantified using the Alpha Imager 2000 system (Alpha Innotech Corp.).

Binding of profilin to monomeric actin was also measured indirectly using an actin polymerization assay. Various concentrations of profilin were mixed with 5 μM G-actin, and polymerization was initiated by the addition of 2 mM MgCl₂, 100 mM KCl at room temperature. After 45 min, the reactions were centrifuged at 90,000 rpm for 20 min in a TLA100 rotor at 25°C. Protein concentration in supernatants and pellets was analyzed by SDS-PAGE as above.

Urea denaturation of profilin was measured by incubating 0.5 μM profilin in PBS buffer containing various concentrations of urea overnight at 4°C. The fluorescence of each protein was monitored at 370-nm emission

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[1]*Abbreviation used in this paper:* PLP, poly-L-proline.

and 280-nm excitation wavelengths. Two independent data sets for each profilin were normalized and averaged. Data was collected on a PTI spectrofluorometer.

The rate of actin treadmilling in the presence of profilin and cofilin was measured by monitoring the release of inorganic phosphate as described (Belmont et al., 1999a).

Results

Actin and Polyproline Binding Functions of Profilin In Vivo

To identify the regions of profilin critical for its function in vivo, we made specific point mutations in conserved amino acid residues located on the yeast profilin surface (Schutt et al., 1993; Eads et al., 1998). Residues predicted to be involved in actin binding or polyproline binding, as well as charged amino acids clustered on the surface of the protein, were replaced with alanine (Fig. 1). Mutant profilin (*pfy1*) alleles were integrated into the endogeneous profilin locus in the yeast genome, and haploid strains were analyzed for growth at various temperatures (Table I). Immunoblotting showed that mutant profilins were expressed at levels indistinguishable from wild-type profilin at both 25 and 37°C (data not shown). We conclude that amino acids in the actin- and polyproline-binding regions of the protein are important for profilin function, whereas charged amino acids located on other surfaces of the protein are not. The phenotypes of all of the mutations were less severe than the profilin deletion, except in the case of *pfy1-12*, which appeared to be lethal.

Actin organization in all mutant strains was examined. Strains with growth defects also displayed disordered actin, the severity of which was proportional to the severity of the growth phenotype. We focused on two mutant pro-
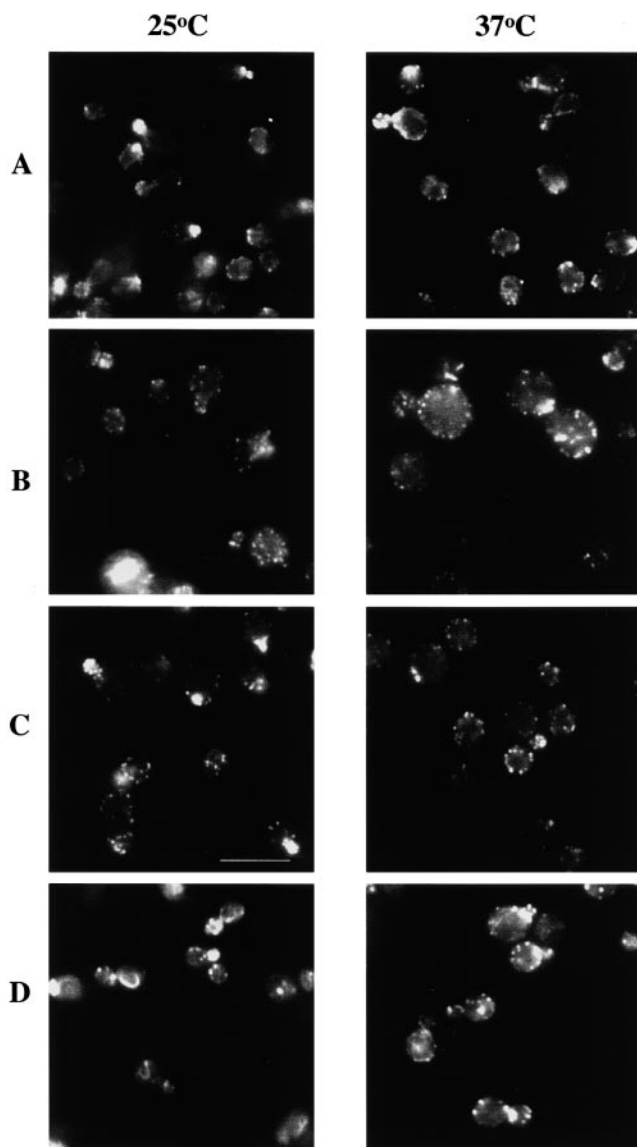
Figure 2. Rhodamine-phalloidin staining of yeast expressing *pfy1-4* and *act1-157*. Yeast cells were grown to log phase in rich media at 25°C then fixed with formaldehyde, or were shifted to 37°C for 3 h, and then fixed. The F-actin was stained with rhodamine phalloidin. Yeast expressing *PFY1* (A), *pfy1-4* (B), *pfy1-4* and *act1-157* (C), and *act1-157* (D). Bar, 10 μm.

filin alleles with severe growth phenotypes, *pfy1-4* and *pfy1-14*, with mutations located in the actin- and polyproline-binding regions, respectively. Actin organization in a *pfy1-4* strain is shown in Fig. 2. The actin phenotype for *pfy1-14* was indistinguishable from that of *pfy1-4* (data not shown). At the permissive temperature of 25°C, strains expressing either *pfy1-4* or *pfy1-14* had a slight defect in localizing actin patches to the bud, and cables were not detectable. These mutant cells were also more round and slightly larger than wild-type cells, and 6% of cells from a log phase culture of *pfy1-4* or *pfy1-14* mutants had more than one bud. When *pfy1-4* or *pfy1-14* cells were shifted to the nonpermissive temperature of 37°C, the cells became more swollen and actin patches became completely nonpolarized, more brightly stained with phalloidin, and more

clumpy. The occurrence of multibudded cells increased to as much as 20%, and in some instances, a thick cable-like structure was observed crossing the length of the cell or curving within the mother cell. The disordered actin cytoskeleton and abnormal morphology of *pfy1-4* and *pfy1-14* are similar to the phenotype of the profilin null (Haarer et al., 1990). Therefore, it appears that the actin- and polyproline-binding regions of profilin are critical for both growth and actin organization in vivo.

Genetic Interactions Provide Evidence for Profilin's In Vivo Function

Although profilin mutagenesis revealed residues critical for function, no phenotypic differences between mutants in the actin- and polyproline-binding regions were observed. To elucidate specific roles for these regions in vivo, yeast strains carrying mutant *pfy1* alleles were crossed to yeast containing mutant actin (*act1*) alleles, and mutant alleles of various cytoskeletal proteins (Table II). The actin mutants chosen for this analysis carry mutations in the nucleotide-binding pocket and differ in their effects on rates of nucleotide exchange and on filament dynamics (Belmont et al., 1999a,b). Nucleotide exchange rates were increased (*act1-157*) or decreased (*act1-158*), or filament dynamics were greatly reduced (*act1-159*). The genetic interactions revealed two aspects of profilin function. First, actin-binding (*pfy1-4*) and polyproline-binding (*pfy1-14*) mutants of profilin can be distinguished genetically. Crossing *pfy1-4* with *act1-157* resulted in the allele-specific suppression of the temperature sensitivity of *pfy1-4*, whereas *pfy1-14* and *act1-157* were synthetic lethal. Second, because Act1-157p exhibits a higher intrinsic rate of nucleotide exchange in vitro compared with wild-type yeast actin (Belmont et al., 1999b), the suppression of the temperature sensitivity of *pfy1-4* by *act1-157* is consistent with the possibility that profilin's ability to catalyze nucleotide exchange on actin

Table II. Crosses between pfy1 Alleles and Mutant Alleles of Cytoskeletal Proteins

	pfy1-4	*pfy1-14*
act1-157	ts at 37°C suppressed	SL
act1-158	SL	SL
act1-159	SL	SL
twf1Δ	SL	SL
srv2Δ	SL	ND
las17ts	NE	ND
abp1Δ	SS	ND
bnr1Δ	SS	ND
arp2-1	NE	ND
	pfy1-4	*pfy1-18*
cof1-4	NE	NE
cof1-5	SL	SS
cof1-6	SS	NE
cof1-19	SL	NE
cof1-22	SL	NE

The result of each cross is listed: ts, temperature sensitivity; SL, synthetic lethal; SS, synthetic sick; NE, no effect; ND, not determined. Crosses were chosen based on the specific properties of these cytoskeletal proteins. Twf1p and Srv2p function as actin monomer sequestering proteins, Las17p is the yeast WASP homologue, and Arp2-1p is the yeast Arp2 homologue in the Arp2/3 complex. All strains listed are available as DDY strains, except *bnr1Δ* and *arp2-1*, which were kindly provided by C. Boone and B. Winsor, respectively.

is important in vivo. Further evidence supporting a role for profilin in actin nucleotide exchange is provided by the observed synthetic lethal interaction between *pfy1-4* and *act1-158*, an actin mutant that displays intrinsically slower rates of nucleotide exchange.

act1-157 Suppresses Actin Cytoskeleton and Fluid Phase Endocytic Defects of pfy1-4 at 37°C

We investigated the nature of the suppression of the temperature sensitivity of *pfy1-4* by *act1-157* by examining actin cytoskeleton organization in these mutant cells. Cells expressing *pfy1-4* at 37°C became very large with depolarized actin patches (Fig. 2 B). In contrast, cells expressing *pfy1-4* and *act1-157* at 37°C (Fig. 2 C) were normal in size and better able to polarize their actin patches to the bud. However, the double mutants still lacked detectable actin cables and were more round than wild-type cells. These results suggest that *act1-157* can suppress some, but not all, of the actin defects of *pfy1-4* at 37°C.

Previously, we have shown that mutations that decrease actin filament turnover also inhibit fluid phase endocytosis (Lappalainen and Drubin, 1997; Belmont and Drubin, 1998). Therefore, we examined fluid phase endocytosis in *pfy1-4* and *act1-157* mutants using Lucifer yellow as a marker. As shown in Fig. 3, yeast expressing *pfy1-4* were defective in Lucifer yellow uptake. At 37°C, only ~18% of cells expressing *pfy1-4* showed detectable Lucifer yellow staining in the vacuole. In contrast, 77% of cells expressing both *pfy1-4* and *act1-157* showed Lucifer yellow staining, a level comparable to staining in wild-type cells (79%) and *act1-157* cells (63%). In addition, of those 18% of *pfy1-4* cells showing Lucifer yellow staining in the vacuole, the staining was consistently weaker in intensity than in double mutant cells (*pfy1-4* and *act1-157*). Interestingly, suppression of the endocytic defects of *pfy1-4* by *act1-157* was not observed at 25°C (Fig. 3). The suppression of actin and endocytic defects of *pfy1-4* by *act1-157* at 37°C suggests that the ability of profilin to enhance nucleotide exchange on actin is critical for fluid phase endocytosis at high temperatures.

The Mutation in pfy1-4 Disrupts Binding to Actin Monomers

To better understand the underlying molecular basis for these observed genetic interactions, we performed a series of biochemical experiments using purified Pfy1-4p and Act1-157p. Three methods were employed. First, we measured the binding of actin to profilin using a PLP cosedimentation assay (polyproline binds to profilin, which in turn binds to actin monomers). As illustrated in Fig. 4 A, wild-type Pfy1p bound to Act1p in a concentration-dependent manner. In contrast, Pfy1-4p was unable to bind Act1p even at 50 μM Pfy1-4p, the highest concentration tested. Next, we measured actin monomer sequestration during actin assembly (Fig. 4 C). Pfy1p sequestered Act1p monomers in a concentration-dependent manner, whereas Pfy1-4p failed to sequester monomers, consistent with its inability to bind to Act1p. The results from these two experiments indicate that Pfy1p binds to Act1p with a K_d of 4–5 μM, consistent with previous reports (Eads et al., 1998). Finally, we examined ATP exchange on actin. Pfy1p
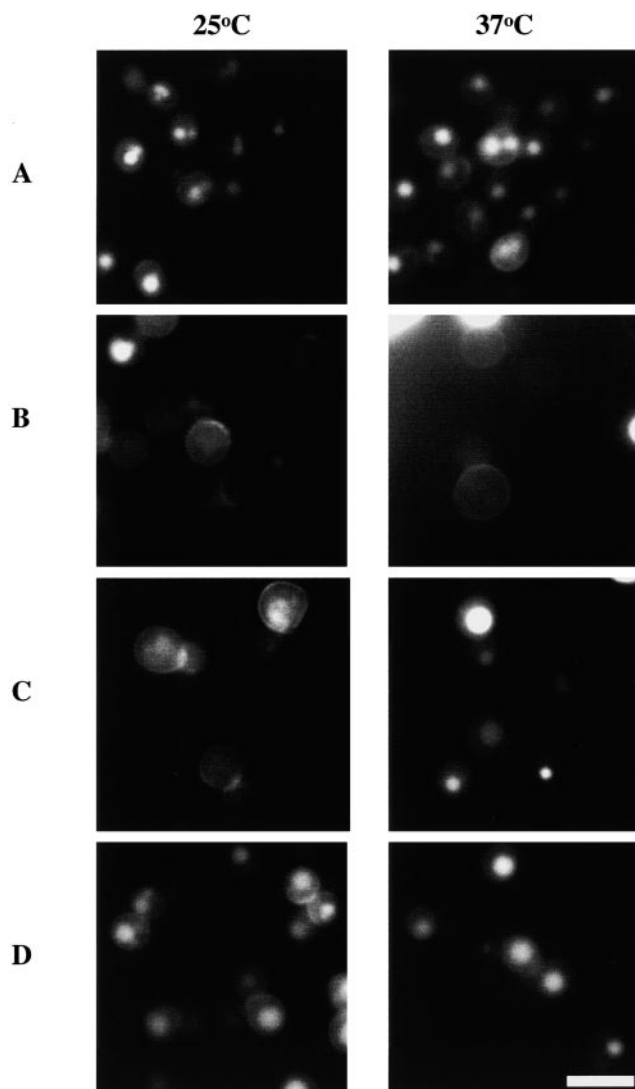

Figure 3. Uptake of Lucifer yellow by fluid-phase endocytosis in yeast expressing *pfy1-4* and *act1-157*. Yeast cells were grown to early log phase at 25°C and some were shifted to 37°C. After 2 h, uptake of Lucifer yellow into the vacuole was monitored by fluorescence microscopy. Yeast expressing *PFY1* (A), *pfy1-4* (B), *pfy1-4* and *act1-157* (C), and *act1-157* (D). Note that in the periphery of B at 37°C, very bright Lucifer yellow staining of dead cells expressing *pfy1-4* is evident. Bar, 10 μm.

was able to increase the rate of Act1p ATP exchange by about threefold, whereas Pfy1-4p had no effect (data not shown). Taken together, these results indicate that Pfy1-4p is unable to bind to Act1p.

To insure that Pfy1-4p is properly folded, we examined its stability in an urea denaturation assay (Fig. 4 D). Pfy1-4p shows a similar denaturation profile to wild-type Pfy1p. In addition, Pfy1-4p binds to PLP with the same affinity as Pfy1p (Fig. 4 A, and data not shown). These results indicate that Pfy1-4p is stable and properly folded, but does not bind to monomeric actin.

Yeast Profilin and Cofilin Cooperate to Increase Turnover of Yeast Actin Filaments

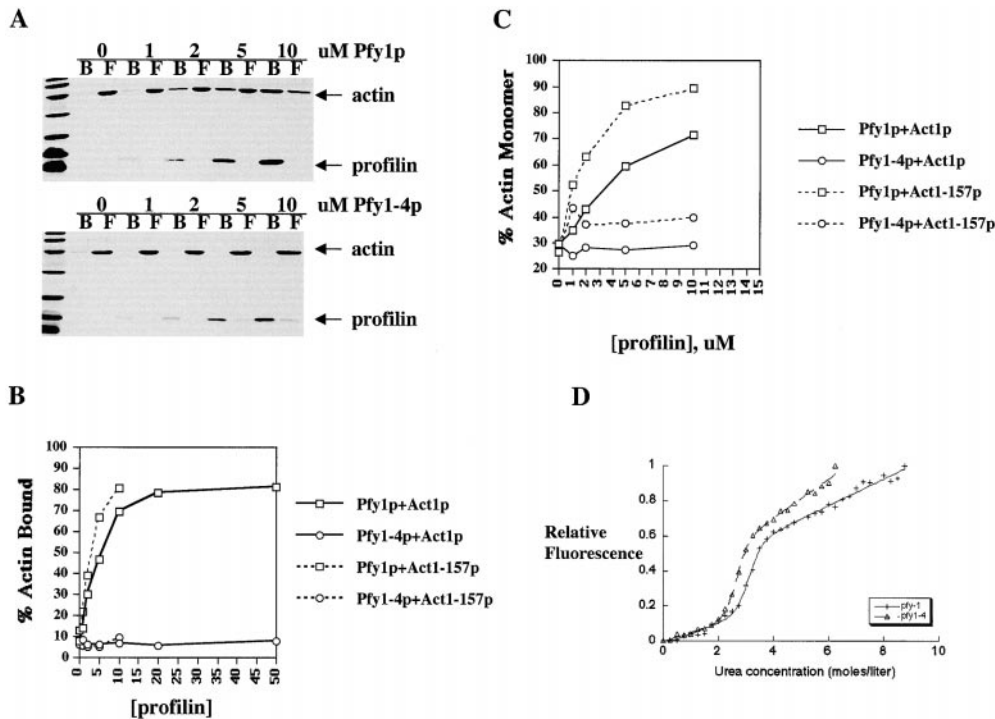The ability of actin to rapidly polymerize and depolymer-

Figure 4. Profilin binding to monomeric actin. Purified yeast actin and recombinant yeast profilin were incubated together to examine actin monomer binding. A, PLP–Sepharose cosedimentation assay. Various concentrations of Pfy1p and Pfy1-4p were bound to PLP–Sepharose and then incubated with 5 μM actin. Actin bound to the beads (B) was separated from the free actin (F) by centrifugation and analyzed by SDS-PAGE and Coomassie staining. B, Quantitation of results from the PLP–Sepharose cosedimentation experiment using various combinations of Act1p, Act1-157p, Pfy1p, and Pfy1-4p. Shown is a plot of the percent of actin bound versus profilin concentration. C, Actin assembly assay. Increasing concentrations of Pfy1p or Pfy1-4p were incubated with 5 μM Act1p or Act1-157p. Steady state reactions were centrifuged, analyzed by SDS-PAGE, and quantitated. D, Urea denaturation of Pfy1p and Pfy1-4p.

ize is critical to its functions in vivo. Cofilin promotes depolymerization from the pointed end of actin filaments, generating ADP-actin monomers, and it inhibits exchange of ATP for ADP. Thus, the ability of profilin to recharge actin monomers with ATP, or alternatively to promote assembly at barbed ends of actin filaments (Pantaloni and Carlier, 1993), may be important for the speed of actin filament turnover when cofilin is present. Indeed, vertebrate profilin and cofilin have been shown to synergize to increase the rate of actin turnover, or treadmilling, in vitro (Didry et al., 1998). We examined the turnover rate of yeast actin filaments in vitro by measuring phosphate released from actin filaments after polymerization in the presence of yeast profilin and cofilin. The combined action of Pfy1p and Cof1p increased the rate of actin filament turnover by approximately fivefold compared with actin alone (Fig. 5 A). In contrast, the rate of actin filament turnover in the presence of Pfy1-4p and Cof1p was similar to a reaction that contained only Cof1p (two- to threefold stimulation). This result is consistent with the observation that Pfy1-4p does not bind to actin. Thus, yeast profilin and cofilin synergize to increase the rate of actin filament turnover in vitro, consistent with published results on vertebrate profilins and cofilins (Didry et al., 1998).

If Pfy1p and Cof1p also act synergistically in vivo to control actin dynamics, then crosses of *pfy1* and *cof1* mutants would be predicted to be synthetic lethal. As shown in Table II, synthetic lethal interactions were observed between *pfy1-4* and several *cof1* alleles. The combined results of these experiments show that synergy between profilin and cofilin on actin turnover are important in vitro and in vivo.

Cofilin Induces Rapid Steady State Turnover of Act1-157 Filaments in the Absence of Profilin

To study the mechanism by which *act1-157* suppresses the temperature sensitivity of *pfy1-4*, we repeated the phosphate release assays using purified Act1-157p. We found that Act1-157p filaments in the absence of profilin or cofilin release phosphate approximately twice as fast as Act1p, suggesting that at steady state nucleotide exchange might be rate-limiting for actin assembly (Fig. 5 B). In the presence of Cof1p, the rate of turnover of Act1-157p filaments is increased to approximately tenfold higher than that of Act1p alone. Pfy1-4p does not alter this increased rate of turnover, consistent with the observation that Pfy1-4p does not bind to Act1-157p (Fig. 4 B). Thus, Act1-157p turns over rapidly in the absence of Pfy1p. This result may explain why *act1-157* is able to suppress the phenotypes of *pfy1-4*.

Two models that might explain our results and the role of profilin in promoting rapid actin filament turnover are: that profilin is required to increase the rate of nucleotide exchange under conditions of rapid turnover (i.e., in the presence of cofilin); or that profilin is required to dissociate ADP-bound actin monomers from cofilin. Although it
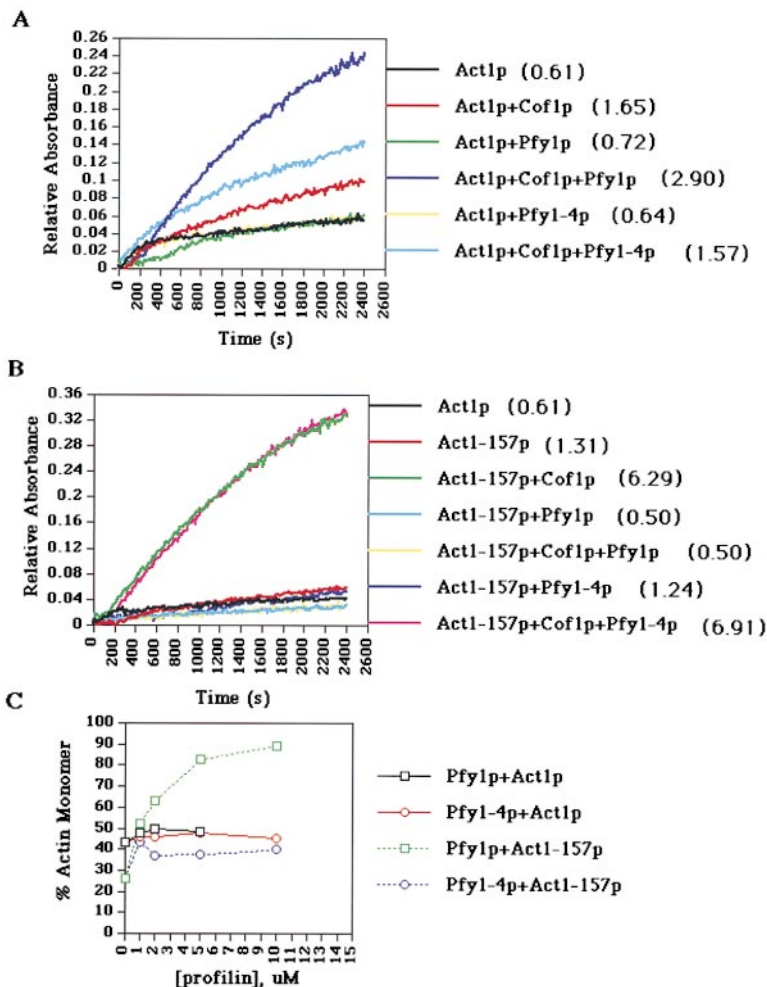
Figure 5. Actin filament turnover assays and steady state assembly. In A and B, purified Act1p, Act1-157p, Pfy1p, Pfy1-4p, and Cof1p were incubated in various combinations at a concentration of 25 μM for each protein. Actin assembly was initiated by the addition of polymerization salts, and phosphate released from actin filaments was measured. Rates of actin treadmilling are measured by comparing the slopes of the linear portions of the graph for each reaction, after steady state levels of assembly have been reached. The numbers in parentheses represent average rates of treadmilling from several experiments, expressed in pmol phosphate/s. A, Actin treadmilling using Act1p. B, Actin treadmilling using Act1-157p. C, Actin assembly in the presence of Pfy1p and Cof1p. Reactions were performed as in Fig. 4 C, except Cof1p was included in each reaction at a concentration of 5 μM.

is difficult to rigorously distinguish between these two models, we favor the first one for the following reasons. First, Act1-157 exhibits a higher intrinsic rate of nucleotide exchange, but no significant differences in polymerization or depolymerization kinetics compared with wild-type actin (Belmont et al., 1999b). If the second model were correct, we would predict that *act1-157* suppresses *pfy1-4* via a mechanism in which ADP-bound Act1-157p has a lower affinity for cofilin, and thus circumvents the need for profilin to dissociate the ADP-actin/cofilin complex. Using the polyproline cosedimentation assay, we indirectly examined the affinity of Cof1p for Act1-157p by testing for the ability of Cof1p to compete with Pfy1p in binding to actin. We found that Cof1p has a similar affinity for ADP-bound wild-type actin as compared with ADP-bound Act1-157p (data not shown). An additional prediction of the second model is that *cof1-19*, which binds less tightly to actin monomers, but has normal disassembly promoting activity on filaments, would at least partially suppress *pfy1-4* (Lappalainen, P., personal communication). However, *cof1-19* is synthetically lethal with *pfy1-4* (Table II). Therefore, we favor a model in which profilin increases actin filament turnover in the presence of cofilin by increasing the exchange of ATP for ADP-bound actin monomers that are released as a result of the actin depolymerizing activity of cofilin.

One initially surprising observation was that, although Act1-157p filaments turn over rapidly in the presence of Cof1p, turnover is extremely slow in the presence of both Cof1p and Pfy1p. This was unexpected because *act1-157* exhibits minor actin defects in vivo. We further investigated the assembly of Act1-157 filaments in the presence of Pfy1p and Cof1p and discovered that Pfy1p tightly sequesters Act1-157p in the presence of Cof1p and prevents its assembly (Fig. 5 C). In addition, based on actin assembly and polyproline cosedimentation assays (Fig. 4, B and C), Pfy1p binds with a higher affinity to Act1-157p (1–2 μM, compared with 4–5 μM for Act1p). The lack of strong actin defects in *act1-157* cells remains a mystery, but may indicate that the monomer-sequestering activity of profilin is regulated by additional proteins in vivo.

Discussion

In this study, we have demonstrated that the actin- and polyproline-binding regions of yeast profilin are critical for its function in vivo. The phenotypes of yeast carrying mutations in these two regions of profilin appeared identical, preventing us from identifying functional differences. Only by performing genetic analysis of double mutants in profilin and actin, and correlating these results with biochemical data on purified mutant proteins, can we con-

clude that one role for profilin in *S. cerevisiae* is to increase the rate of actin filament turnover. Our studies support the hypothesis that nucleotide exchange on actin can be a rate-limiting step for actin assembly during rapid actin rearrangements in vivo (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991; Vinson et al., 1998; Selden et al., 1999). One function of profilin may be to provide a pool of ATP actin competent to assemble quickly onto filament barbed ends in response to internal or external signals. Indeed, profilin is important for achieving maximal rates of actin-based motility of *Listeria* bacteria (Loisel et al., 1999). Yeast require rapid actin filament turnover for efficient fluid phase endocytosis (Lappalainen and Drubin, 1997; Belmont and Drubin, 1998). In this study, we observed that endocytosis is defective in yeast carrying a mutant profilin that cannot bind to actin, and is rescued at high temperature by an actin mutant that displays high rates of nucleotide exchange and filament turnover independent of profilin. These results strongly suggest that, through its ability to catalyze nucleotide exchange on actin, profilin promotes actin filament dynamics in vivo.

An important aspect of the experiments presented here is the use of both profilin and cofilin in actin filament turnover assays. Cofilin increases the rate of actin turnover by promoting depolymerization, yet it actually decreases the rate of nucleotide exchange on actin (Blanchoin and Pollard, 1998). The combination of cofilin's ability to depolymerize actin filaments and decrease the rate of actin nucleotide exchange would be predicted to generate a large pool of ADP actin. Profilin's ability to enhance exchange on actin might therefore be necessary to maximize actin turnover in the presence of cofilin. Indeed, profilin has been shown to overcome the inhibition of nucleotide exchange by the cofilin-related protein, actophorin (Blanchoin and Pollard, 1998), and we have obtained similar results with yeast profilin, cofilin, and actin (Wolven, A.K., and D.G. Drubin, unpublished results). Including both profilin and cofilin in the actin filament turnover assays more accurately reflects the in vivo situation, and strengthens the conclusion that actin nucleotide exchange can be a rate-limiting step that requires profilin in vivo.

An alternative hypothesis to explain the results obtained here is that the role of profilin is to dissociate cofilin from ADP actin, rather than promote nucleotide exchange. While our results would suggest that this is not the case, we cannot rule out a model in which profilin dissociates ADP actin from cofilin by catalyzing ATP exchange on actin. It is possible that dissociation of ADP actin-cofilin and nucleotide exchange occur in a concerted manner and are mechanistically coupled.

In contrast to vertebrate and amoeba profilins, plant profilins do not appear to enhance the rate of nucleotide exchange on actin, although rabbit muscle actin was used in the studies of plant profilin (Perelroizen et al., 1996). In yeast, profilin only modestly increases the rate of ATP exchange on actin (Eads et al., 1998). Such results have led to speculation that in plants and yeast, profilin may play other roles in actin dynamics. Our results indicate that the ability of profilin to promote nucleotide exchange on actin is important in *S. cerevisiae*. However, profilin has additional activities in vitro and our results also suggest that other actin related and unrelated functions of profilin

must be important in vivo. Evidence for this conclusion comes from the observations that *act1-157* does not completely alleviate the slow growth and disrupted actin organization in yeast expressing *pfy1-4*, and that *pfy1-4* yeast grow better than *pfy1Δ* yeast. Other important activities of profilin might include promoting barbed-end assembly, preventing pointed-end assembly, sequestering actin monomers to inhibit spontaneous nucleation, and modulating lipid chemistry (Goldschmidt-Clermont et al., 1991; Pantaloni and Carlier, 1993; Kang et al., 1999). The context under which actin is assembling in vivo may determine which actin-related activity of profilin is used. Evidence that profilin may have other activities is also provided by the synthetic lethal interactions observed between *pfy1-4* and *srv2Δ*, and between *pfy1-4* and *twf1Δ* (Table II). Srv2p and Twfp can both sequester actin monomers (Freeman et al., 1995; Goode et al., 1998). These double mutants may have problems regulating actin monomer pools.

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