

Interactions with PIP₂, ADP-actin monomers, and capping protein regulate the activity and localization of yeast twinfilin

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Twinflin is a ubiquitous actin monomer-binding protein that regulates actin filament turnover in yeast and mammalian cells. To elucidate the mechanism by which twinfilin contributes to actin filament dynamics, we carried out an analysis of yeast twinfilin, and we show here that twinfilin is an abundant protein that localizes to cortical actin patches in wild-type yeast cells. Native gel assays demonstrate that twinfilin binds ADP-actin monomers with higher affinity than ATP-actin monomers. A mutant twinfilin that does not interact with actin monomers *in vitro* no longer localizes to cortical actin patches when expressed in yeast, suggesting that the ability to interact with actin

monomers may be essential for the localization of twinfilin. The localization of twinfilin to the cortical actin cytoskeleton is also disrupted in yeast strains where either the *CAP1* or *CAP2* gene, encoding for the α and β subunits of capping protein, is deleted. Purified twinfilin and capping protein form a complex on native gels. Twinfilin also interacts with phosphatidylinositol 4,5-bisphosphate (PI[4,5]P₂), and its actin monomer-sequestering activity is inhibited by PI(4,5)P₂. Based on these results, we propose a model for the biological role of twinfilin as a protein that localizes actin monomers to the sites of rapid filament assembly in cells.

Introduction

The actin cytoskeleton underlies multiple cell biological processes including endocytosis, secretion, morphogenesis, motility, and cell division. Although actin filaments represent the functional state of actin in most cell biological processes, the actin monomer pool also plays an important role in cytoskeletal dynamics. After dissociation from the pointed end of the actin filament, ADP-actin monomers can be sequestered in the cytoplasm or localized to areas of rapid actin filament assembly. After exchange of ADP for ATP, actin monomers can add to one end of a preexisting filament or participate in the nucleation of a new actin filament (Sheterline et al., 1998).

A number of actin monomer-binding proteins regulate the size and dynamics of the actin monomer pool in cells. However, only three classes of small actin monomer-binding pro-

teins are found in organisms as diverse as yeast and mammals (Lappalainen et al., 1998): cofilin/actin-depolymerizing factors (ADFs),* profilins, and twinfilins.

Cofilin/ADF proteins promote the addition of actin monomers to the cytoplasmic pool by depolymerizing (and severing) preexisting actin filaments (Carlier et al., 1997; Lappalainen and Drubin, 1997; Rosenblatt et al., 1997) and interacting with actin monomers. Under physiological conditions, cofilin/ADFs have an \sim 100-fold higher affinity for ADP- than for ATP-actin monomers (Maciver and Weeds, 1994; Carlier et al., 1997), and they inhibit the nucleotide exchange of the actin monomers (Hawkins et al., 1993).

In contrast to cofilin/ADF proteins, profilins have a higher affinity for ATP- than for ADP-actin monomers and promote nucleotide exchange on actin monomers (Goldschmidt-Clermont et al., 1991; Vinson et al., 1998). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, profilin's ability to enhance nucleotide exchange on actin monomers *in vivo* is important (Wolven et al., 2000; Lu and Pollard, 2001). Profilin can also promote filament assembly because profilin-actin complexes can add to barbed ends (Pantaloni and Carlier, 1993). Furthermore, profilin binding to monomers suppresses the spontaneous nucleation of actin filaments, functioning as an actin

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*Abbreviations used in this paper: ADF, actin-depolymerizing factor; GFP, green fluorescent protein; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.

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monomer–sequestering protein in the absence of free filament ends (Vinson et al., 1998).

Twinfilin is a 35–40-kD actin monomer–binding protein that was originally identified from the budding yeast *S. cerevisiae* (Goode et al., 1998). Homologues of twinfilin have been found in *S. pombe*, *Caenorhabditis elegans*, mice, and humans, suggesting that twinfilins are present across the entire spectrum of eukaryotic organisms (Vartiainen et al., 2000). Twinfilins are composed of two domains homologous to cofilin/ADF proteins (ADF-H domain) that are separated by a short linker. Unlike cofilin/ADF proteins, twinfilin only interacts with actin monomers and inhibits the assembly of actin filaments in a stoichiometric manner in vitro (Goode et al., 1998; Vartiainen et al., 2000). Furthermore, twinfilin inhibits the nucleotide exchange on actin monomers (Goode et al., 1998). Deletion of the twinfilin gene in yeast results in abnormal cortical actin patches, defects in bipolar bud site selection pattern, and synthetic lethality with certain cofilin and profilin mutations (Goode et al., 1998; Wolven et al., 2000). Overexpression of twinfilin in yeast and mouse cells results in the formation of abnormal actin structures (Goode et al., 1998; Vartiainen et al., 2000). These findings suggest that twinfilin, together with cofilin/ADF and profilin, is involved in the regulation of the dynamics of the actin cytoskeleton. However, the mechanism by which twinfilin contributes to actin filament turnover is not understood.

Here we show that twinfilin is an abundant protein, that it binds ADP-actin preferentially, and that it localizes to cortical actin patches in yeast. The localization of twinfilin to actin patches is disrupted by mutations at the actin monomer–binding site of twinfilin. Furthermore, localization of twinfilin to actin patches depends on the presence of the actin filament barbed-end capping protein, Cap1/2p. We suggest that twinfilin localizes ADP-actin monomers at sites of rapid actin filament assembly in cells; therefore, it may serve as a link between actin filament depolymerization and actin filament assembly.

Results

Yeast twinfilin preferentially binds ADP-actin monomers

Purified yeast and mouse twinfilins bind actin monomers in a 1:1 ratio and prevent the assembly of these monomers into actin filaments (Goode et al., 1998; Vartiainen et al., 2000). To elucidate how twinfilin contributes to actin filament turnover, it is important to understand whether it binds ADP- or ATP-actin monomers with higher affinity. We studied the interaction of purified yeast twinfilin with yeast actin monomers using a native gel electrophoresis assay. As shown in lanes 1 and 2 of Fig. 1, twinfilin and actin display different mobilities in a 7.5% native polyacrylamide gel. However, when mixed with each other before loading on a gel, a clear shift in the mobility of twinfilin and ADP-actin monomers is observed. Instead of two independent bands, these two proteins migrate in one band, resulting in different mobility as compared with these proteins alone (Fig. 1 B, lanes 3–5). This suggests that twinfilin and ADP-actin monomers form a stable complex with each other under

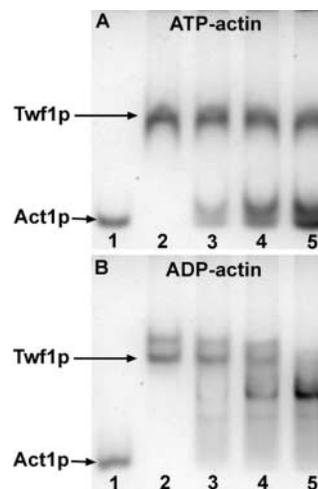


Figure 1. Twinfilin forms a stronger complex with ADP-actin monomers than with ATP-actin monomers. Binding of Twf1p to yeast actin monomers was studied by a native gel electrophoresis assay in which Twf1p was loaded on a gel either separately or as a mixture with actin. The actin on gel A was in ATP form, and the gel and buffers contained 50 μ M ATP. The actin on gel B was hydrolyzed to ADP form by hexokinase treatment and the buffers and gel contained 50 μ M ADP. The concentration of twinfilin was 10 μ M (lanes 2–5), whereas the actin concentrations on lanes 3–5 were 5, 10, and 15 μ M, respectively. Lane 1 shows the mobility of 10 μ M actin alone. Lanes 4 and 5 on panel B show a strong complex formation between actin and twinfilin, indicating that twinfilin forms a stable complex with Mg^{2+} -ADP-actin monomers.

these buffer conditions. The complex formation between twinfilin and ATP-actin monomers is much weaker than the one observed with ADP-actin monomers, indicating that twinfilin interacts with ATP-actin monomers with a significantly lower affinity than with ADP-actin monomers (Fig. 1). The double band seen in twinfilin (Fig. 1 B, lanes 2 and 3) may result from twinfilin's interaction with the nucleotide. However, this does not appear to affect twinfilin's ability to bind actin monomer (Fig. 1 B, lane 5).

Yeast twinfilin is an abundant protein

To understand the role of twinfilin for actin filament turnover in vivo, it is important to know the abundance of twinfilin in cells; therefore, we raised polyclonal antibodies against purified yeast twinfilin. On Western blots, the affinity-purified antibodies recognize one polypeptide with an identical mobility to that of recombinant twinfilin, indicating that these antibodies are specific and that twinfilin in yeast does not have any major posttranslational modifications. A Western blot analysis with three known concentrations of purified actin, cofilin, and twinfilin, as well as three different dilutions of wild-type yeast extracts, showed that the actin:cofilin:twinfilin ratio in these yeast extracts is \sim 10:2.5:1 (Fig. 2). This shows that twinfilin is an abundant protein in yeast cells.

Localization of twinfilin in yeast cells

In mouse cell lines, twinfilin shows strong cytoplasmic staining concentrated at cortical actin filament structures that are rich in actin monomers and/or filament ends as visualized by DNaseI staining (Vartiainen et al., 2000). Because of the lack of anti-

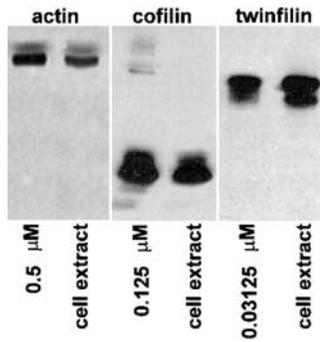


Figure 2. Twinfilin is an abundant protein in yeast cells. Yeast cell extracts as well as known concentrations of actin, cofilin, and twinfilin were run on 12% polyacrylamide gels and the proteins were subsequently visualized by Western blotting. By comparing the intensities of the protein bands in the cell extracts with the purified protein samples we estimated that the actin/cofilin/twinfilin ratio in yeast cells is $\sim 10:2.5:1$.

bodies against yeast twinfilin, localization of this protein in yeast cells was previously studied only by using twinfilin green fluorescent protein (GFP) fusion proteins. In wild-type yeast cells, the twinfilin–GFP fusion protein showed diffuse cytoplasmic localization, although cortical patch-like staining was also occasionally observed (Goode et al., 1998). However, at least in mammalian cells, GFP appears to disturb the localization of twinfilin because GFP fusion protein localizes diffusely to the cytoplasm, whereas endogenous twinfilin also localizes to the cortical actin cytoskeleton (Vartiainen et al., 2000).

We studied the localization of twinfilin in yeast cells by using the affinity-purified polyclonal antibodies described in the legend to Fig. 2. These antibodies are also specific for twinfilin in immunofluorescence because a twinfilin deletion strain shows no specific staining (Fig. 3 C). In a wild-type yeast strain, twinfilin shows cytoplasmic staining but it is also strongly colocalized with the cortical actin patches (Fig. 3, A and B). No twinfilin staining could be detected on the cytoplasmic actin cables.

To study whether the localization of twinfilin to the cortical actin cytoskeleton in yeast cells is dependent on the intact actin cytoskeleton, we depolymerized actin filaments from yeast cells with the drug latrunculin-A. A complete and reversible disruption of the actin cytoskeleton in yeast cells can be induced by this actin filament assembly inhibitor (Ayscough et al., 1997). After a 20-min incubation with 500 μM latrunculin-A, both actin and twinfilin showed diffuse cytoplasmic localization (Fig. 3, E and F), suggesting that the localization of twinfilin to the cortical actin patches is dependent on the presence of intact actin filaments.

Mutations in the actin-binding site affect the localization of twinfilin

To understand whether twinfilin's interaction with an actin monomer is required for localization, we designed a mutant twinfilin that did not interact with actin monomers. In designing such a mutant, we took advantage of the sequence homology between the ADF-H domains of twinfilin and cofilin/ADF proteins. Based on our multiple sequence alignments, residues R88 and 90 in the NH_2 -terminal, and resi-

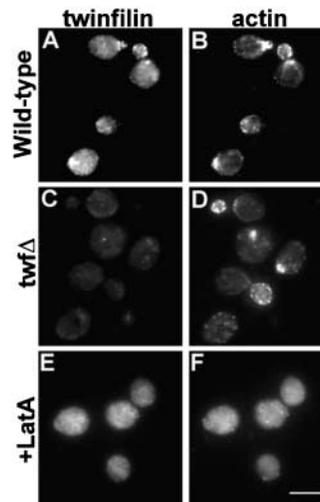


Figure 3. Twinfilin localizes to cortical actin patches in yeast cells. Indirect immunofluorescence microscopy was performed on wild-type (DDY1102), *twfΔ* (DDY1436), and wild-type yeast cells treated with 500 μM latrunculin-A for 20 min. Twinfilin and actin in these cells were visualized with affinity-purified rabbit anti-twinfilin antibody (A, C, and E) and guinea pig anti-actin antisera (B, D, and F). In wild-type cells (A and B), twinfilin shows relatively strong cytoplasmic staining but is also concentrated to cortical actin patches. In *twfΔ* cells (C and D), no twinfilin staining could be observed. In wild-type cells treated with latrunculin-A (E and F), both twinfilin and actin show diffuse localization. Bar, 5 μm .

duces K254 and R256 in the COOH-terminal ADF-H domain of yeast twinfilin, correspond to residues that are crucial for actin binding in cofilin/ADFs (Moriyama et al., 1992; Lappalainen et al., 1997). Therefore, we chose to replace these residues by alanines either in the NH_2 -terminal ADF-H domain (Twf1-1p), the COOH-terminal ADF-H domain (Twf1-2p), or in both ADF-H domains (Twf1-3p).

Purified wild-type and mutant twinfilins were analyzed for actin monomer interactions by actin filament sedimentation and native gel electrophoresis assays. In actin filament sedimentation assays, we compared the ability of wild-type and mutant twinfilins to shift actin from filaments (pellet) to the monomer pool (supernatant). Wild-type twinfilin efficiently shifts actin from filaments to the monomer pool with the monomer-sequestering activity saturated at 4 μM twinfilin. Twf1-1p, in which only residues in the first ADF-H domain are replaced by alanines, is somewhat less efficient in sequestering actin monomers in this assay. Also, Twf1-2p shows detectable actin monomer sequestering activity, but it is significantly less efficient than Twf1-1p in shifting actin into the monomeric fraction. Twf1-3p, in which residues in both ADF-H domains are mutated, can no longer shift detectable amounts of actin into the supernatant fraction with the protein concentration range probed in this study (Fig. 4 A). The defect in actin monomer interactions was also seen in native gel electrophoresis assays. Whereas wild-type twinfilin forms a stable complex with ADP-actin monomers on native gels, no detectable complex formation between Twf1-3p and ADP-actin monomers could be observed (unpublished data). The far UV CD spectra of purified wild-type and Twf1-3p twinfilins are almost identical to each other,

Table I. Yeast strains used in this study

Strain	Genotype
BGY11	MAT α , his3, leu2, ura3, ade2, trp1, lys2, crn1 Δ ::LEU2
DAY32	MAT a, leu2 Δ 1, ura3-52, trp1 Δ 63, his3 Δ 200, aip1 Δ ::URA3
DDY196	MAT α , ura3-52, tpm1::LEU2, his3-Del200, ade2, leu2
DDY318	MAT α , Δ sac6::LEU2, his3 Δ 200, leu2-3,112, lys2-801, ura3-52, GAL+
DDY322	MAT α , his3 Δ 200, leu2-3,112, ura3-52, Δ abp1::LEU2
DDY333	MAT α , his3 Δ 200, ura3-52, Δ sla1::URA3
DDY546	MAT α , his3 Δ 200, leu2-3,112, lys2-801, sla2- Δ 1::URA3, ura3-52
DDY950	MAT α , ura3-52, leu2-3,112, lys2-801, trp1-1, Δ rvs167::TRP1
DDY952	MAT α , his3 Δ 200, leu2-3,112, ura3-52, lys2-801, srv2 Δ 2::HIS3
DDY1102	MAT a/MAT α , ade2-1/+, his3 Δ 200/his3 Δ 200, leu2-3,112/leu2-3,112, ura3-52/ura3-52, lys2-801/+
DDY1266	MAT α , ura3-52, his3 Δ 200, leu2-3,112, lys2-801, cof1-22::LEU2
DDY1436	MAT a/MAT α , ade2-1/ade2-1, his3 Δ 200/his3 Δ 200, leu2-3,112/leu2-3,112, ura3-52/ura3-52, Δ twf1::URA3/ Δ twf1::URA3
KKY62	MAT a, his3 Δ 200, ura3-52, lys2-801, cdc42-1
MDY26	MAT a, lys2-801, ura3-52, leu2-3,112, his3 Δ 200, las17 Δ ::URA3
PLY13	MAT α , ura3-52, his3 Δ 200, leu2-3,112, lys2-801, ade2-101, COF1::LEU2
PLY15	MAT α , ura3-52, his3 Δ 200, leu2-3,112, lys2-801, cof1::LEU2
T65.1D	MAT α , leu2-3,112, ade1, ura3-52, lle-, MEL1, vrp1::LEU2
YJC0388	MAT a, rho+, ade2-101, his3-11,15, leu2-3,112, trp1-1, ura3-1
YJC0389	MAT a, rho+, ade2-101, his3-11,15, leu2-3,112, trp1-1, ura3-1, cap2- Δ 1::HIS3
YJC0390	MAT a, rho+, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, cap1::TRP1
YJC0391	MAT α , rho+, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, cap1::TRP1, cap2::HIS3

suggesting that the composition of secondary structure elements in these proteins is very similar (Fig. 4 B). Additionally, the mutations in Twf1-3p do not decrease the protein

stability because wild-type twinfilin has a T_m value of 55°C, whereas the T_m value of Twf1-3p is 59°C.

To examine whether the localization of twinfilin to the cortical actin cytoskeleton depends upon its ability to bind actin monomers, we expressed wild-type and Twf1-3p mutant twinfilin in a *twf1 Δ* yeast strain under a GPD promoter. Based on our quantitative Western blot assays, both wild-type and mutant twinfilin are expressed in approximately tenfold higher levels with this expression system, as compared with wild-type cells in which twinfilin is expressed under a control of its own promoter (unpublished data). However, the expression levels varied from cell to cell; therefore, we could probe the localization of wild-type and mutant twinfilins in cells where their expression levels were similar to wild-type yeast cells. As shown in Fig. 5, A and B, wild-type twinfilin localizes mainly to the cortical actin patches, whereas Twf1-3p shows diffuse cytoplasmic localization (Fig. 5, C and D). This result was also confirmed by constructing GFP fusion proteins of wild-type and Twf1-3p twinfilins. When expressed in yeast cells, the Twf1p-GFP localized to cortical patch-like structures, whereas Twf1-3p-GFP showed diffuse cytoplasmic localization (unpublished data).

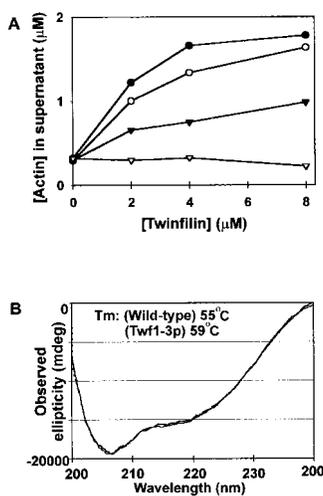


Figure 4. **The mutant twinfilin Twf1-3p is not able to bind actin monomers.** (A) The ability of wild-type (●), Twf1-1p (○), Twf1-2p (▲), and Twf1-3p (△) to sequester actin monomers is demonstrated by a graphical representation of the quantitation of the amount of actin present in the supernatant fraction in actin filament sedimentation assays. The actin concentration in the supernatant was 4 μ M and the twinfilin concentration varied from 0 to 8 μ M. Wild-type twinfilin efficiently increases the amount of yeast actin in the supernatant, whereas Twf1-3p is much less efficient in increasing the amount of actin in the supernatant. (B) Wild-type twinfilin and Twf1-3p have similar structure and stability. Far UV CD spectra of wild-type twinfilin (solid line) and Twf1-3p (dotted line) at 20°C are almost identical. Twf1-3p appears to be slightly more stable than wild-type twinfilin, as judged on the basis of melting points that were measured by following α helix distortion by CD signal at 222 nm as the temperature was raised in intervals of 5°.

Interaction with Cap1/2p is required for localization of twinfilin

Yeast twinfilin is an actin monomer-binding protein, and is therefore expected to localize to the cytoplasm in yeast cells. However, as shown in Fig. 3, in addition to diffuse cytoplasmic localization, twinfilin is also found in cortical actin patches. Furthermore, its localization to the cortical actin patches is dependent on the intact actin cytoskeleton (Fig. 3, E and F). These data indicate that the localization of twinfilin depends on additional components at the cortical actin patch. Furthermore, the localization of the putative twinfilin ligand at the cortical actin patch must be dependent on an intact actin cytoskeleton. Among the known actin patch components,

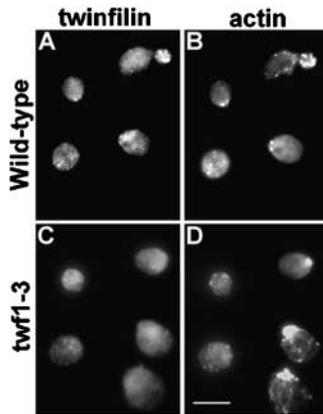


Figure 5. A mutant twinfilin Twf1-3p that is no longer able to bind actin monomers does not localize to cortical actin patches. Wild-type twinfilin and Twf1-3p were expressed in *twfΔ* cells (DDY1436) and twinfilin (A and C) and actin (B and D) were visualized. Wild-type twinfilin (A and B) localizes to cortical actin patches, whereas Twf1-3p (C and D) shows diffuse cytoplasmic localization. Bar, 5 μ m.

such proteins include Cof1p, Aip1p, Sac6p, Crn1p, Abp1p, Srv2p, and Cap1/2p (for review see Pruyne and Bretscher, 2000). To test if any of these proteins would be responsible for the localization of twinfilin to the cortical actin patches, we studied the localization of twinfilin in yeast strains carrying deletions or mutations in the genes encoding for these proteins. We also tested twinfilin localization in some other yeast mutant strains in which other known components and regulators of the actin cytoskeleton are mutated (Tables I and II).

With three exceptions, twinfilin localized normally to the cortical actin cytoskeleton in these yeast strains (Table II). The first mutant strain in which twinfilin showed abnormal localization carries a deletion of the *LAS17* gene. This gene encodes for the yeast homologue of Arp2/3 activator WASP (Li, 1997). In these cells the actin cytoskeleton was severely disturbed and twinfilin localized only to cortical actin patch-like structures, whereas no twinfilin staining could be

Table II. Localization of twinfilin to the actin patches in various mutant strains

Mutations in yeast strains	Localization
Wild type	+ ^a
<i>crn1Δ::LEU2</i>	+
<i>cap2-Δ1::HIS3</i>	- ^b
<i>cap1-Δ1::TRP1</i>	-
<i>aip1Δ::URA3</i>	+
<i>vrp1Δ::LEU2</i>	+
<i>srv2-Δ2::HIS3</i>	+
<i>Δtmp1::LEU2</i>	+
<i>sla2-Δ1::URA3</i>	+
<i>Δsac6::LEU2</i>	+
<i>Δrvs167::TRP1</i>	+
<i>cdc42-1</i>	+
<i>las17Δ::URA3</i>	+
<i>Δabp1::LEU2</i>	+
<i>Δsla1::URA3</i>	+
<i>cof1-22::LEU2</i>	+

^aColocalization between actin and twinfilin.

^bNoncolocalization.

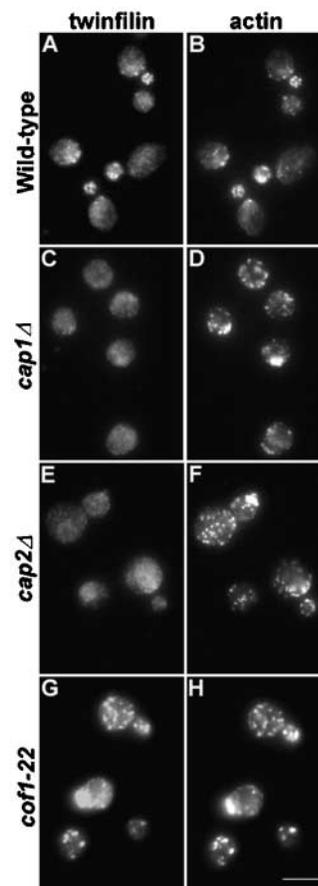


Figure 6. The presence of intact capping protein, Cap1/2p, is required for localization of twinfilin to cortical actin patches. The localization of twinfilin (A, C, E and G) and actin (B, D, F and H) was examined in wild-type (A and B), *cap1Δ* (C and D), *cap2Δ* (E and F), and *cof1-22* (G and H) yeast strains. Twinfilin colocalizes with the cortical actin patches in wild-type and *cof1-22* strains, whereas twinfilin shows diffuse cytoplasmic localization in *cap1Δ* and *cap2Δ* strains. Bar, 5 μ m.

detected in large actin bundles (unpublished data). Li (1997) reported that the abnormal actin bundles in the *las17Δ* strain are not structurally related to actin patches, because they do not contain most of the cortical cytoskeletal components. Therefore, the lack of twinfilin localization to these actin bundles in the *las17Δ* strain is probably an indirect effect that results from the abnormalities in the composition of these actin filament structures.

Two other yeast strains in which twinfilin showed an abnormal localization are *cap1Δ* and *cap2Δ*. These carry deletions of the α and β subunits of actin filament barbed-end capping protein, Cap1/2p. In these cells, twinfilin localized diffusely in the cytoplasm, and stronger staining was only occasionally observed at and/or near cortical actin patches (Fig. 6, C–F). Whereas virtually all actin patches were positive for twinfilin staining in the wild-type parent strain, detectable patch-like twinfilin staining could be observed in <3% of the actin patches in the *cap2Δ* strain. Based on a quantitative Western blot analysis, the *cap2Δ* cells have the same level of twinfilin as wild-type cells, demonstrating that the lack of twinfilin localization to the cortical actin patches in the *cap2Δ* strain is not a result of decreased twinfilin levels (un-

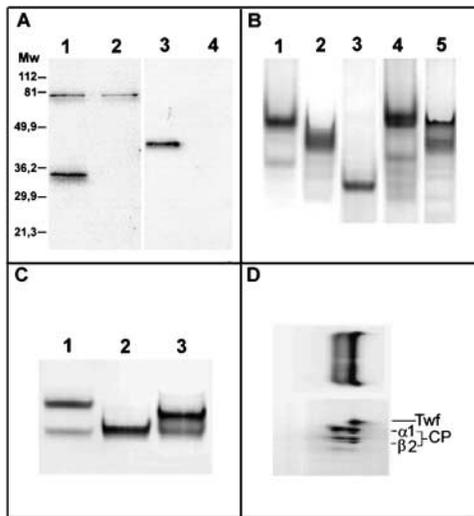


Figure 7. Twinfilin interacts with capping protein. (A) Immunoprecipitation of twinfilin with anti-yeast twinfilin antibody was carried out from wild-type (lane 1) and $\Delta twf1$ (lane 2) yeast extracts. The blot detected with an anti-Cap2p antibody shows coimmunoprecipitation of Cap2p (MW ~ 33 kD) with twinfilin from wild-type yeast extract. Immunoprecipitation of Cap2p was carried out with anti-Cap2p antibody from wild-type (lane 3) and $\Delta cap1, \Delta cap2$ (lane 4) strains. The blot detected with an anti-Twf1p antibody shows specific coimmunoprecipitation of twinfilin (MW ~ 40 kD) with capping protein. The ~ 80 -kD band seen in lanes 1 and 2 is a protein that binds unspecifically to protein A-Sepharose beads and cross-reacts with the Cap2p antiserum. (B) The interaction between yeast twinfilin and yeast capping protein was also investigated by native gel electrophoresis. Lane 1, 10 μ M twinfilin; lane 2, 10 μ M Twf1-3p; lane 3, 5 μ M Cap1/2p; lane 4, 10 μ M twinfilin + 5 μ M Cap1/2p; lane 5, 10 μ M Twf1-3p + 5 μ M Cap1/2p. The shift in mobility of Cap1/2p in the presence of twinfilin or Twf1-3p indicates a complex formation. (C) Interaction of mouse twinfilin and mouse $\alpha 1\beta 2$ capping protein. Lane 1, 3 μ M twinfilin; lane 2, 3 μ M capping protein; lane 3, 3 μ M twinfilin + 3 μ M capping protein. (D) Analysis in a second dimension on a 12% SDS-polyacrylamide gel of the protein components from B (lane 3) shows the presence of twinfilin and both subunits ($\alpha 1$ and $\beta 2$) of capping protein in the shifted band.

published data). The defect in actin patch localization in the *cap2* Δ strain is also specific for twinfilin because other actin patch components, such as cofilin and Abp1p, localize normally to cortical actin patches in the *cap2* Δ strain (unpublished data). These results show that Cap1/2p is required for efficient localization of twinfilin to actin patches. Twinfilin also localizes to the cortical actin patches in the *cof1-22* strain (Fig. 6, G and H). In this yeast strain, the actin monomer pool is depleted due to a mutation in the actin filament depolymerizing protein cofilin (Lappalainen and Drubin, 1997). Therefore, it is unlikely that the localization defect observed in *cap1* Δ and *cap2* Δ strains results from a decrease in the cytoplasmic actin monomer pool in the absence of Cap1/2p.

An interaction between twinfilin and Cap1/2p was further examined by a coimmunoprecipitation assay. Cap1/2p coimmunoprecipitates with anti-Twf1p antibody in the presence, but not in the absence, of twinfilin (Fig. 7 A, lanes 1 and 2). Similarly, twinfilin coimmunoprecipitates with anti-Cap2p antibody (Fig. 7 A, lanes 3 and 4), suggesting that these proteins interact with each other in vivo. A native PAGE assay was car-

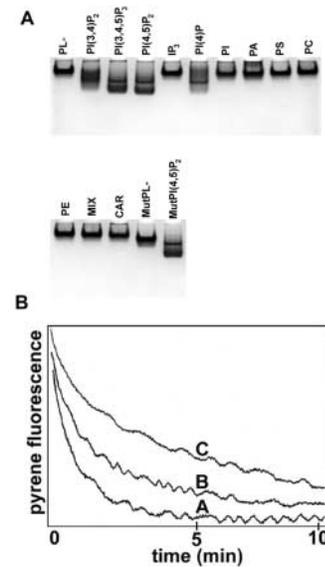


Figure 8. Twinfilin interacts with PIPs. (A) Native gel electrophoresis was performed to detect binding of wild-type twinfilin to different phospholipids or phospholipid headgroups. PI(4,5)P₂ and PIP(3,4,5)P₃ and, to a lesser extent, PI(3,4)P₂ and PI(4)P, cause twinfilin to move more rapidly towards the anode indicating a net increase in the negative charge and thus a binding interaction. PL- stands for twinfilin sample without any phospholipid, whereas IP₃, PI, PA, PS, PC, PE, MIX, and CAR denote inositol(1,4,5) trisphosphate headgroup, phosphatidylinositol, phosphatidic acid, phosphatidylserine, -choline, -ethanolamine, lipid mixture (cholesterol, lecithin, and lysolecithin), and cardiolipin, respectively. Also the mutant twinfilin, Twf1-3p [mut PL-], interacts with PI(4,5)P₂ in this assay [mut P(4,5)P₂]. (B) PIP₂ inhibits the actin monomer-sequestering activity of twinfilin. Actin filaments (6 μ M, 1:5 pyrene rabbit actin: yeast actin) were polymerized for 20 min. Depolymerization was induced by mixing 40 μ l of actin with 10 μ l of 25 μ M twinfilin that had been incubated with PI(4,5)P₂ for 5 min. The depolymerization of filaments was followed by the decrease in the fluorescence at 407 nm. The final concentrations of PI(4,5)P₂ were 0 μ M (A.), 25 μ M (B.) and 50 μ M (C.). The presence of 50 μ M PI(4,5)P₂, and to a lesser extent 25 μ M PI(4,5)P₂, result in a decrease in the rate and extent of F-actin depolymerization.

ried out to elucidate whether purified twinfilin and capping protein interact with each other in vitro. Purified Cap1/2p runs as a single band below the migration position of yeast twinfilin on a native gel (Fig. 7 B). However, when mixed with each other before loading on gel, a shift in the mobility of Cap1/2p is observed, suggesting that the two proteins form a complex. Also, Twf1-3p mutant twinfilin interacts with Cap1/2p in this assay (Fig. 7 B). We also studied the interaction of purified mouse twinfilin and mouse capping protein ($\alpha 1\beta 2$) with this assay. Mixing these two proteins with each other prior the loading on a gel results in a formation of an intermediate mobility band between the original migration positions of these proteins (Fig. 7 C). Based on a Coomassie blue-stained second dimension SDS-PAGE, this complex contains a 1:1:1 molar ratio of twinfilin: $\alpha 1$ subunit: $\beta 2$ subunit (Fig. 7 D).

Interactions with PIP₂ inhibit the actin monomer-sequestering activity of twinfilin

Next, we carried out a native gel electrophoresis assay to examine whether twinfilin interacts with phospholipids. In

this assay, twinfilin was loaded on a native polyacrylamide gel either alone or in a mixture with various phospholipids and then run towards the anode by its own charge. As shown in Fig. 8 A, purified yeast twinfilin (PL-) migrates as a single sharp band in this gel. When mixed with PI(4)P, PI(3,4)P₂, PI(4,5)P₂, or PIP(3,4,5)P₃ before loading on a gel, twinfilin migrates as a smear. Twinfilin appears to be able to differentiate between various phosphatidyl inositols, because the smear-formation with PI(4,5)P₂ and PI(3,4,5)P₃ is somewhat more intense than with PI(3,4)P₂ and PI(4)P. Interestingly, IP₃, which is the polar head group of PI(4,5)P₂, does not have any effect on twinfilin mobility on this native gel electrophoresis assay (Fig. 8). This result suggests that in addition to the polar head group, the fatty acid side chains of PI(4,5)P₂ are also essential for interactions with twinfilin.

To examine the effects of PI(4,5)P₂ for the actin-binding activity of twinfilin, we monitored the ability of twinfilin to depolymerize pyrene-labeled actin filaments in the presence and absence of PI(4,5)P₂. With the concentrations used in this study, PI(4,5)P₂ does not significantly affect the fluorescence signal. Purified yeast twinfilin induces a rapid depolymerization of actin filaments by its ability to sequester actin monomers as they dissociate from the ends of the filament. The reaction reaches a steady-state level ~ 3 min after the addition of twinfilin on pyrene-labeled filaments (Fig. 8 B). This activity is similar to the one induced by actin monomer-sequestering drugs such as latrunculin-A (see, for example, Goode et al., 1998). In the presence of PI(4,5)P₂, twinfilin is much less efficient in sequestering actin monomers than in the absence of phospholipids. For example, with 50 μ M PI(4,5)P₂, the reaction is not completed after 10 min of addition of twinfilin-PI(4,5)P₂ mixture on actin filaments.

Discussion

Different actin monomer-binding proteins differ in their relative affinities for ADP- and ATP-actin monomers. Previous studies have showed that cofilin/ADF proteins, which also bind and depolymerize actin filaments, have a several-fold higher affinity for ADP- than for ATP-actin monomers at physiological buffer conditions (Carrier et al., 1997; Res-sad et al., 1998). Profilin, which in cells enhances the rate of nucleotide exchange on actin monomers, promotes filament assembly at barbed ends, and suppresses spontaneous actin filament nucleation (Pantaloni and Carrier; 1993; Vinson et al., 1998; Wolven et al., 2000), has an approximately three- to fourfold higher affinity for ATP- than ADP-actin. Thymosin β 4, an actin monomer-sequestering protein found in higher eukaryotes, also preferentially binds ATP-actin monomers over ADP-actin monomers (Vinson et al., 1998). Here, we report that yeast twinfilin forms a more stable complex with ADP- than with ATP-actin monomers. Because of difficulties in labeling yeast actin with NBD, we have so far been unable to determine the exact dissociation constants between yeast twinfilin and yeast actin monomers. However, our recent studies with mouse twinfilin have showed that its affinity for ADP-actin monomers at physiological salt is ~ 50 nM, whereas the affinity for ATP-actin monomers is ~ 400 nM (unpublished data). Therefore,

twinfilin binds ADP-actin monomers with a several-fold higher affinity than ATP-actin monomers, and has a different preference for the nucleotide state of the actin monomer than profilin and thymosin β 4. These results indicate that twinfilin also sequesters actin monomers through a different mechanism than these two other actin monomer-binding proteins. Interestingly, the preference for ADP-actin monomers over ATP-actin monomers is similar between cofilin/ADF proteins (Carrier et al., 1997) and twinfilin, suggesting that the ADF-H domain may be a specific ADP-actin-binding domain.

Twinfilin is an abundant protein in yeast, found in an $\sim 1:10$ ratio to actin and a 1:2.5 ratio to cofilin. Because twinfilin appears to have a high affinity for ADP-actin monomers, our results suggest that it can bind and/or sequester a significantly large proportion of actin monomers in yeast cells at any given time. The abundance of profilin in yeast cells has not been reported, but in *Acanthamoeba* cells, profilin is found in a 1:2 ratio to actin (Tseng et al., 1984). However, it is important to note that the actin monomer pool in yeast *S. cerevisiae* cells has been reported to be very small compared with the one in more motile organisms and their cell types (Karpova et al., 1995). In contrast to profilin, twinfilin prefers to interact with ADP-actin monomers and inhibits the nucleotide change upon binding (Goode et al., 1998); therefore, it may have a significant effect on the size and nucleotide status of the actin monomer pool in yeast cells.

The actin cytoskeleton in yeast is composed of two types of filamentous structures: cortical actin patches and cytoplasmic actin cables. These two cytoskeletal structures have a different, but somewhat overlapping, composition of actin-binding proteins. Furthermore, the dynamics of these two cytoskeletal domains are different; patches are more dynamic and composed of shorter filaments than cables (Karpova et al., 1998). Twinfilin shows diffuse cytoplasmic staining, but is also concentrated to cortical actin patches. We did not observe any twinfilin staining in actin cables, although it is possible that twinfilin staining on faint actin cables would not be visible above the background (Fig. 3). Localization of twinfilin to actin filament structures is unexpected, because twinfilin binds actin monomers in vitro.

To study whether twinfilin needs to be associated with an actin monomer to localize to patches, we constructed a mutant twinfilin that was unable to bind actin monomers. A twinfilin mutant (Twf1-3p) in which two key charged residues in both ADF-H domains are replaced by alanines is no longer able to bind and sequester actin monomers. These residues correspond to the most critical actin monomer-binding residues (R96 and K98) in yeast cofilin. Therefore, cofilin/ADF proteins and the ADF-H domains in twinfilin appear to interact with actin through overlapping interfaces. Interestingly, mutations in the COOH-terminal ADF-H domain (Twf1-2p) have a stronger effect on actin monomer sequestering activity than the mutations in the NH₂-terminal ADF-H domain (Twf1-1p) (Fig. 4 A), suggesting that the former domain may be more important for actin monomer interactions. The mutant twinfilin (Twf1-3p) interacts with Cap1/2p and PI(4,5)P₂, suggesting that these binding

sites do not overlap with the actin-binding site (Figs. 7 and 8). When expressed in yeast cells under a GPD promoter or as a GFP fusion protein under a Gal promoter, wild-type twinfilin localized at the cortical actin patches, whereas Twf1-3p showed diffuse cytoplasmic localization. These results suggest that, in order to localize at the cortical actin cytoskeleton, twinfilin may require the ability to associate with an actin monomer. It is possible that the interaction with an actin monomer promotes a conformational change in the three-dimensional structure of twinfilin or in the orientation of the two ADF-H domains with respect to each other, increasing the affinity of the twinfilin-actin monomer complex for its ligand at the cortical actin patches.

The localization of twinfilin to the cortical actin cytoskeleton is dependent on actin filaments (Fig. 3, E and F). Therefore, we speculated that the localization of twinfilin's binding partner at the cortical actin patches would also be actin dependent. From the currently known yeast actin-binding proteins, this criterion narrows the number of proteins to seven (for review see Pruyne and Bretscher, 2000). Twinfilin showed normal cortical actin patch localization in all but two of the mutant strains carrying deletions or mutations in these and some other known actin patch associated proteins (Table II.). These exceptions are *cap1Δ* and *cap2Δ* strains, which carry deletions of the subunits of capping protein, Cap1/2p. It has been shown that a deletion of the gene for either of the capping protein subunits leads to a loss of also the other subunit (Amatruda et al., 1992). This implies that twinfilin requires the presence of intact capping protein, Cap1/2p, to localize to the cortical actin filament structures in yeast cells. Twinfilin and capping protein also interact with each other in native gel assays, suggesting that a direct interaction between these two proteins may promote the localization of twinfilin to cortical actin cytoskeleton. However, the interaction between twinfilin and Cap1/2p can be also detected in the absence of actin monomers *in vitro*, whereas a mutation (Twf1-3p) that disrupts the actin-binding site of twinfilin no longer localizes at the cortical actin patches *in vivo*. It is possible that Twf1-3p mutation also has some other, currently unidentified, defects that prevent its correct localization in cells. Alternatively, interaction with an actin monomer may increase the affinity of twinfilin for capping protein, and may therefore be essential for the correct localization of twinfilin *in vivo*. Because there are currently no quantitative methods available for determining the affinities of twinfilin for capping protein in presence and absence of actin, we cannot distinguish between these two alternatives.

The activity of several actin-binding proteins is regulated by PIPs *in vitro*. As shown in Fig. 8, A and B, yeast twinfilin binds PI(4,5)P₂ and this interaction downregulates its actin monomer-sequestering activity. Therefore, the interaction with PIP₂ may serve as a mechanism to prevent twinfilin from sequestering actin monomers at the regions of rapid actin filament nucleation and assembly in cells.

Fig. 9 shows a hypothetical model for the function of twinfilin in yeast cells. ADP-actin monomers dissociate from the minus end of the filament either spontaneously or by a cofilin/ADF-stimulated mechanism. Because twinfilin and cofilin interact with actin monomers through overlapping

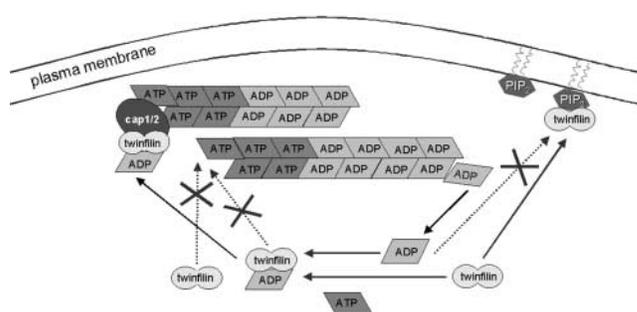


Figure 9. A hypothetical model for the localization and function of twinfilin in yeast cells. Twinfilin prefers to interact with ADP-actin monomers over ATP-actin monomers. Twinfilin also binds PIP₂, and its actin monomer-binding activity is inhibited by these PIP₂-interactions. The localization of twinfilin to cortical actin filament structures may require its ability to interact with an actin monomer. Furthermore, the localization of twinfilin to cortical actin patches is dependent on the presence of an intact Cap1/2p. The function of twinfilin may be to localize ADP-actin monomers at the sites of rapid actin filament assembly in cells.

interfaces (Fig. 4), we speculate that twinfilin can sequester the actin monomer from cofilin. Twinfilin inhibits the spontaneous nucleotide exchange on the actin monomer (Goode et al., 1998). Therefore, it is probably able to keep the actin monomer in ADP form and prevent the assembly of this monomer into a filament. The function of twinfilin may be to transport actin monomers, in their inactive ADP form, to cortical actin patches. The possible function of twinfilin as an actin monomer-localizing protein is supported by the synthetic lethality between *twf1*-null mutation and specific cofilin (*cof1-22*) and profilin (*pfj1-4*) mutations. In *cof1-22* cells, the actin monomer pool is depleted due to defects in actin filament depolymerization (Lappalainen and Drubin, 1997). In *pfj1-4* cells, the exchange of actin nucleotide form ADP to ATP is defective due to mutations in profilin (Wolven et al., 2000). In combination with a possible defect in actin monomer localization in *twf1Δ* cells, either one of these mutations would be expected to result in a dramatic decrease in the amount of ATP-actin monomers at the sites of rapid actin filament assembly in cells.

The localization of a twinfilin-actin monomer complex to the cortical actin filament structures requires the presence of an intact Cap1/2p, and twinfilin also interacts with Cap1/2p *in vitro*. Our preliminary experiments indicate that Cap1/2p and twinfilin do not affect each other's activities (unpublished data). In the future, it will be important to examine whether the ADP-actin monomer dissociates spontaneously from twinfilin after localization to the cortical actin cytoskeleton, or whether the dissociation is assisted by other proteins, such as profilin. It is also somewhat confusing why twinfilin interacts with capping protein, because in motile processes the role of capping protein is to cap the barbed ends of filaments and block the assembly of actin monomers into these filaments (Loisel et al., 1999). At least in the leading edge of migrating cells and in *Listeria* actin tails, capping protein is expected to localize behind the actual actin filament assembly zone. However, it is important to remember that yeasts are nonmotile organisms and that their cortical actin patches are small structures composed of short actin

filaments (Karpova et al., 1998); therefore, yeast capping protein is located close to the actin filament assembly zone.

In conclusion, yeast twinfilin is an abundant ADP-actin monomer-binding protein that localizes to the cortical actin cytoskeleton. The localization of twinfilin at the cortical actin filament structures appears to be dependent on the interaction with an actin monomer. Twinfilin also interacts with Cap1/2p, and the presence of intact capping protein is required for its localization to the cortical actin cytoskeleton. Therefore, twinfilin may function as a protein that links the actin filament depolymerization to filament assembly by localizing actin monomers to the sites of rapid actin filament assembly.

Materials and methods

Site-directed mutagenesis and plasmid construction

The site-directed mutations were introduced to yeast twinfilin cDNA by using the PCR-based overlap extension method (Higuchi et al., 1988). The oligonucleotides used in amplification created NcoI and HindIII sites at the 5' and 3' ends of the final PCR fragments, respectively. These fragments were ligated into an NcoI-HindIII-digested pGAT2 plasmid (Peränen et al., 1996) backbone to create plasmids pPL71, 75, and 105. The constructs were then sequenced by the chain termination method to verify the correct sequence. The plasmids for expressing wild-type and Twf1-3p mutant twinfilins in yeast were constructed by ligating the PCR fragments into a NotI-SpeI-digested pHCA plasmid, a gift from Kathryn Ayscough (University of Glasgow, Glasgow, UK) to create plasmids pPL77 (wild type) and 76 (*twf1-3*).

Protein expression and purification

Wild-type and mutant yeast twinfilins were expressed and purified as described (Goode et al., 1998). Actin was purified from yeast cell extracts by DNaseI affinity chromatography as described by Rodal et al. (1999). Yeast cofilin and mouse twinfilin were purified as described by Lappalainen et al. (1997) and Vartiainen et al. (2000), respectively. Yeast Cap1/2p protein was expressed and purified as described by Amatruda et al. (1992). Skeletal muscle actin was purified from chicken pectoral muscle as described by Spudich and Watt (1971), and pyrene actin was prepared as described by Cooper et al. (1983).

The plasmid for the expression of mouse capping protein $\alpha 1$ and $\beta 2$ subunits was constructed in a pET3d vector (pET3d[m- $\alpha 1/\beta 2$]) using the strategy described by Soeno et al. (1998) for chicken capping protein ($\alpha 1\beta 1$). Mouse $\alpha 1\beta 2$ capping protein was expressed and purified from BL21(DE3) *Escherichia coli*. One liter of LB media containing carbenicillin ($50 \mu\text{g}/\text{ml}^{-1}$) was grown shaking at 37°C until the $A_{600\text{nm}}$ was between 0.6 and 1.0. Expression was induced by the addition of IPTG to 1 mM and by growth for 3 h. Cells were harvested by centrifugation ($3000 g$ for 15 min), resuspended, and washed once in 100 ml of 40 mM TrisCl, pH 8.0, 40 mM EDTA, and 140 mM NaCl. The cell pellet was resuspended in 100 ml of ice-cold 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, and sonicated on ice for $12 \times 10\text{-s}$ bursts. The cell lysate was subjected to centrifugation in a Beckman Coulter Ti45 rotor at 35,000 rpm for 1 h at 4°C . A 50–70% ammonium sulphate fraction was obtained from the high-speed supernatant, and the precipitate obtained by centrifugation at $32,000 g$ for 20 min at 4°C . The pellet was resuspended in 40 ml of ice-cold HA buffer (10 mM KH_2PO_4 , pH 7.0, 500 mM KCl, 1 mM DTT, 1 mM PMSF, 0.01% NaN_3), dialyzed overnight against HA buffer, and applied to a hydroxyapatite column ($2.5 \times 10\text{ cm}$) equilibrated in HA buffer. Proteins were eluted with a 10–250-mM KH_2PO_4 gradient in HA buffer and analyzed by SDS-PAGE. Fractions containing capping protein were pooled and dialyzed against Q buffer (10 mM TrisCl, pH 8.0, 10 mM KCl, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.01% NaN_3). The dialyzed sample was loaded onto a Mono Q column ($2.6 \times 10\text{ cm}$) equilibrated in Q buffer. Proteins were eluted with a 10–400-mM KCl gradient in Q buffer. Fractions containing capping protein were pooled and dialyzed against SA buffer (10 mM MES, pH 6.0, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.01% NaN_3) followed by dialysis for 1.5 h against SB buffer (10 mM MES, pH 5.8, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.01% NaN_3). The sample was loaded onto a Mono-S column ($2.6 \times 10\text{ cm}$) equilibrated in SB buffer. The column was eluted with a 0–350-mM NaCl gradient in SB buffer, and the fractions containing capping protein were pooled, concentrated, and dialyzed against 10 mM TrisCl, pH 8.0, 40 mM KCl, 0.5 mM DTT, and 50% glycerol for storage at -70°C .

Native gel electrophoresis assays

Native PAGE for studying the twinfilin-actin monomer interaction was performed as described by Safer (1989) and as modified by Maciver and Weeds (1994). ADP-actin was prepared by incubating ATP-actin with agarose-linked yeast hexokinase (Sigma-Aldrich) for 3 h at 4°C in the presence of 1 mM glucose and 0.2 mM EGTA (Pollard, 1986). Native PAGE to study protein-lipid interactions was performed as described by Gungabissoon et al. (1998). The tissue-extracted lipids with heterogeneous fatty acid composition were purchased from Sigma-Aldrich, with the exception of PI(3,4)P₂, PI(3,4,5)P₃, polar lipid mix, cardiolipin, and phosphatidic acid that were from Matreya, Inc.

Twinfilin-Cap1/2p interaction was studied on 10% native polyacrylamide gels. Purified yeast and mouse capping protein and twinfilin (either alone or in a mixture with each other), was diluted to the desired concentrations in 10 mM Tris, pH 7.5, 50 mM NaCl, and 0.5 mM DTT, and incubated for 60 min at room temperature. A 20- μl aliquot was then mixed at a ratio of 4:1 with loading buffer (125 mM Tris, pH 9.0, 250 mM NaCl, 2.5 mM DTT, 50% glycerol) and then loaded onto the gel. The gel was run at 120 V for 100 min with native running buffer (25 mM Tris, 194 mM glycine, pH 9.0, 0.5 mM DTT). For analysis in a second dimension with SDS-PAGE, an entire lane from the first dimension native gel was excised using a razor blade. The edges of the lane were trimmed to remove the "smile" section of the gel. The gel piece was then placed horizontally $\sim 5\text{ mm}$ from the top of a clean glass plate. The gel lane was then incubated in $\sim 2\text{--}3\text{ ml}$ of SDS-PAGE running buffer and $\sim 1\text{ ml}$ of 10% SDS for 15 min at room temperature. The excess liquid was carefully removed, the gel piece clamped between two glass plates, and a 12% (wt/vol) SDS-polyacrylamide separating gel carefully poured underneath. After this had set, a 4% (wt/vol) SDS-polyacrylamide stacking gel was poured around the gel piece, completely covering it. Protein standard lanes were also placed in the stacking gel.

Actin filament sedimentation and depolymerization assays

Actin filament sedimentation assays were carried out as described by Goode et al. (1998). The concentration of actin was constant ($4\ \mu\text{M}$) and twinfilin was used in final concentrations of 0, 2, 4, and $8\ \mu\text{M}$. Kinetics of actin filament disassembly was monitored by pyrene fluorescence with excitation at 365 nm and emission at 407 nm. $6\ \mu\text{M}$ actin (5:1, yeast actin pyrene-labeled rabbit skeletal muscle actin; Cytoskeleton, Inc.) was polymerized in F buffer for 30 min. Disassembly of F-actin was induced by mixing 40 μl of F-actin with 10 μl of 25- μM yeast twinfilin, and monitored by the decrease in fluorescence at 407 nm for 10 min in Hitachi F-4010 fluorescence spectrophotometer. Twinfilin was pre-incubated for 5 min with PI(4,5)P₂ before mixing with F-actin. The final concentrations of PI(4,5)P₂ in this assay were 0, 25, and $50\ \mu\text{M}$.

Immunofluorescence microscopy

Cells were grown in appropriate medium at 30°C to an optical density of 0.5 at 600 nm, and prepared for immunofluorescence as described by Ayscough and Drubin (1998). The antibody against yeast twinfilin was raised by immunizing rabbits with the purified recombinant first ADF-H domain (residues 1–162). The antibodies were then affinity purified from the rabbit antiserum with the same protein. The anti-actin antiserum was generated by immunizing guinea pigs with purified yeast actin. The guinea pig anti-yeast actin serum was used at a dilution of 1:1,000, and the rabbit anti-yeast twinfilin antibody was used at a dilution of 1:50. Latrunculin-A (Molecular Probes) was used at a final concentration of $500\ \mu\text{M}$ for 20 min at 37°C .

Western blotting and coimmunoprecipitation

Cells were grown to confluence overnight in YEPD medium (1% [wt/vol] Bacto yeast extract, 2% Bacto peptone, 2% glucose) at 30°C , diluted (1:10), and allowed to grow $\sim 3\text{ h}$, after which cells from a 3-ml cell culture were spun down and resuspended in 100 μl 20 mM Tris-HCl, pH 7.5, 0.6 mM PMSF, and protease-inhibitor cocktail (1:1,000) (500 μg each of antipain, leupeptin, pepstatin, chymostatin, and aprotin per ml; Sigma-Aldrich). Cells were lysed by adding glass beads (1:1) and vortexing for 5 min at room temperature. Western blotting was carried out as described by Vartiainen et al. (2000) with the following primary antibodies: rabbit anti-yeast actin (1:500), cofilin (1:500), or twinfilin (1:1,000). Coimmunoprecipitation experiment using 10^8 cells of DDY1102, DDY1436, YJC0388, and YJC0391 yeast strains was carried out as described (Paunola et al., 1998). The primary antibodies (rabbit anti-Twf1p and guinea pig anti-Cap2p) were covalently coupled to protein A-Sepharose beads. Twinfilin and capping protein were visualized from Western blots with rabbit anti-Twf1p (1:1,000) and guinea pig anti-Cap2p (1:10,000) antibodies.

CD spectroscopy

CD measurements were recorded with a Jasco J-700 spectropolarimeter equipped with a microcomputer and a Jasco PTC-348WI thermostat. Spectra were collected with a scan speed of 50 nm/min, step resolution of 0.2 nm, bandwidth of 2.0 nm, sensitivity of 20 millidegrees, and with a response time of 1 s. Each spectrum was the average of at least 20 scans. Far UV CD spectra were recorded at a protein concentration of 3 μ M in 2 mM NaPO₄, pH 7.4, and 25 mM UV-free NaCl (Sigma-Aldrich) with a 2-mm pathlength optical cell. For temperature transition studies, six scans at the desired temperature were recorded after an incubation time of 4 min in a given temperature, and the distortion of α helices (Yang et al., 1986) was plotted at 222 nm.

Miscellaneous

PAGE was carried out by using the buffer system described by Laemmli (1970). Protein concentrations were determined with Hewlett Packard 8452A Diode Array Spectrophotometer by using calculated extinction coefficients for yeast twinfilin (at 280 nm ϵ = 11.6 mM⁻¹ cm⁻¹), yeast actin (at 290 nm –320 nm ϵ = 26.6 mM⁻¹ cm⁻¹), cofilin (at 280 nm ϵ = 14.7 mM⁻¹ cm⁻¹), and mouse capping protein (α 1 β 2) (at 280 nm ϵ = 76.3 mM⁻¹ cm⁻¹). The concentration of yeast Cap1/2p was quantified from Coomassie blue-stained SDS gels compared with purified yeast twinfilin and actin. Protein distributions in SDS-PAGE gels were quantified by Fluor-S™ Multilimager with Quantity One (v. 4.1.0; Bio-Rad Laboratories) or TINA (v. 2.09c) software.

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