Regulation of the Cortical Actin Cytoskeleton in Budding Yeast by Twinfilin, a Ubiquitous Actin Monomer-sequestering Protein

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Abstract. Here we describe the identification of a novel 37-kD actin monomer binding protein in budding yeast. This protein, which we named twinfilin, is composed of two cofilin-like regions. In our sequence database searches we also identified human, mouse, and *Caenorhabditis elegans* homologues of yeast twinfilin, suggesting that twinfilins form an evolutionarily conserved family of actin-binding proteins. Purified recombinant twinfilin prevents actin filament assembly by forming a 1:1 complex with actin monomers, and inhibits the nucleotide exchange reaction of actin monomers. Despite the sequence homology with the actin filament depolymerizing cofilin/actin-depolymerizing factor (ADF) proteins, our data suggests that twinfilin does not induce actin filament depolymerization. In yeast cells, a green fluorescent protein (GFP)–twinfilin fusion protein localizes primarily to cytoplasm, but also to cortical actin patches. Overexpression of the twinfilin gene (*TWF1*) results in depolarization of the cortical actin patches. A *twf1* null mutation appears to result in increased assembly of cortical actin structures and is synthetically lethal with the yeast cofilin mutant *cof1-22*, shown previously to cause pronounced reduction in turnover of cortical actin filaments. Taken together, these results demonstrate that twinfilin is a novel, highly conserved actin monomer-sequestering protein involved in regulation of the cortical actin cytoskeleton.

Key words: actin • cytoskeleton • budding yeast • twinfilin • cofilin

THE actin cytoskeleton plays an essential role in multiple cellular processes, including polarized cell growth, cell motility, cytokinesis, secretion, and endocytosis. All of these processes rely on the capacity of the actin cytoskeleton to respond to cellular signals and reorganize spatially and temporally into a variety of specific structures. To regulate the organization and turnover of the actin cytoskeleton, a number of actin filament- and actin monomer-binding proteins have evolved in eukaryotic organisms (for recent review, see Carlier and Pantaloni, 1997). Most families of actin-binding proteins are highly conserved throughout evolution and are found in organisms as diverse as humans and yeast. This suggests that these proteins existed previously in a common eukaryotic ancestor and that the basic mechanisms for the regulation of the actin cytoskeleton dynamics are conserved among diverse organisms and cell types.

Several different families of actin-binding proteins can directly regulate the nucleation and elongation of actin filaments. The evolutionarily conserved Arp2/3 complex is composed of two actin-related proteins and five to six other subunits (Machesky et al., 1994). This protein complex nucleates actin polymerization by serving as a template for the formation of new actin filaments (Welch et al., 1997). On the other hand, actin filament barbed-end capping proteins of the capZ family regulate elongation of actin filaments by blocking the addition of actin monomers to the barbed-end of the filament (Hug et al., 1995). Actin filament depolymerizing proteins of the actin-depolymerizing factor (ADF)[1]/cofilin family regulate actin filament depolymerization and turnover by increasing the dissociation rate of actin monomers from the pointed end in vitro and in vivo (Carlier et al., 1997; Lappalainen and Drubin, 1997; Rosenblatt et al., 1997). Cofilin, together with the actin monomer binding proteins thymosin-β-4 and profilin, helps to maintain a pool of actin monomers that is available for new filament assembly (Carlier and Pantaloni, 1997). Thymosin-β–4 functions as a strong actin monomer-sequestering protein, whereas cofilin and profilin do not sequester subunits, but

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1. *Abbreviations used in this paper*: ADF, actin-depolymerizing factor; GFP, green fluorescent protein.

appear to promote subunit addition at the barbed-end (Pantaloni and Carlier, 1993; Carlier et al., 1997). Although cofilin and profilin are highly conserved in evolution from yeast to mammals, thymosin-β-4 has to date only been found in animal cells (Sun et al., 1995), and is absent in the *S. cerevisiae* genome (P. Lappalainen and B.L. Goode, unpublished data). Thus, a high affinity actin-sequestering protein that is evolutionarily conserved has not yet been identified.

Here we describe the identification and characterization of a novel, highly conserved actin monomer binding protein. This protein is composed of two cofilin-like regions and was therefore named twinfilin. Biochemical characterization of twinfilin shows that this protein sequesters actin monomers by forming a 1:1 molar complex with actin. Furthermore, in vivo analyses of the *TWF1* gene in budding yeast showed that twinfilin is involved in the regulation of the cortical actin cytoskeleton.

Materials and Methods

TWF1 Deletion

The *TWF1* gene was deleted from the wild-type diploid strain DDY1102 (Table I) using a PCR-based one-step gene replacement technique (Baudin et al., 1993). The *URA3* gene was amplified from pRS316 plasmid (Sikorski and Hieter, 1989) using oligonucleotides that introduce 50-bp *TWF1* 5′ and 3′ flanking sequences to the ends of the *URA3* gene. The PCR products were gel purified and transformed into DDY1102 to yield integrative recombinants that were selected for by growth on medium lacking uracil. Correct integration was confirmed by PCR amplification of genomic DNA from URA^+ cells with primers external to the *TWF1* gene.

Immunofluorescence and Localization of a GFP–Twinfilin Fusion Protein in Yeast

Cells were prepared for immunofluorescence as described by Ayscough and Drubin (1998). The guinea pig anti–yeast actin serum was used at 1:2,000 dilution and the rabbit anti–GFP serum, a gift from Pam Silver (Dana Farber Cancer Institute, Harvard University), was used at 1:10,000 dilution. Cells were viewed using a Zeiss Axioskop fluorescence microscope with a 100 W mercury lamp and a Zeiss 100X Plan-NeoFluar oil immersion objective. Images were captured electronically using a 200-E CCD camera (Sony Electronics Inc., San Jose, CA) and displayed on a Micron 133 computer (Micron Electronics Inc., Nampa, ID) using Northern Exposure software (Phase 3 Imaging Systems, Milford, MA).

A GFP-*TWF1* gene fusion was generated by PCR amplifying the *TWF1* coding region using primers that introduce HindIII and XbaI sites at the 5′ and 3′ ends, respectively. The PCR product was ligated into the GFP vector, pTS408 (a gift from Tim Stearns, Stanford University, Stanford, CA), which had been digested with HindIII and XbaI.The resulting plasmid, pGFP-TWF1, was transformed into haploid (DDY757; Mat a) and diploid (DDY759; Mat a/α) yeast strains (Table I). To induce expression of the GFP–twinfilin fusion protein, cultures were grown in selective synthetic medium plus glucose to log phase (OD_{600} = 0.1), pelleted, and transferred to synthetic selective medium plus 2% galactose for 12 h at 25°C. Live cells were mounted on slides and imaged by fluorescence microscopy.

Bud-Scar Staining and Endocytosis Assay

The budding patterns of *TWF1/TWF1* (DDY1102) and *Δtwf1/Δtwf1* (DDY1436) diploid cells were scored after calcofluor staining as described in Yang et al. (1997). For each strain, >200 cells with 3 or more visible scars were scored. Fluid phase endocytosis was assayed as described (Dulic et al., 1991) by monitoring uptake of the dye lucifer yellow to the vacuole.

Overexpression of Twinfilin in Yeast

To overexpress twinfilin in yeast, the entire *TWF1* gene coding region was amplified by PCR using primers that introduce HindIII and KpnI sites at the 5′ and 3′ ends, respectively. The PCR product was ligated in frame into the gal-inducible *CEN/URA3* vector, pRB1438 (a gift from the Botstein lab, Stanford University) digested with HindIII and KpnI, and the resulting plasmid, p*GAL-TWF1*, transformed into DDY757 cells (Table I). To induce overexpression of twinfilin, cultures were grown in selective synthetic medium plus glucose to log phase (OD_{600} = 0.1), pelleted, and transferred to synthetic selective medium plus 2% galactose for 24 h at 25°C. The effects of overexpression on the actin cytoskeleton were examined by actin immunofluorescence in parallel with cells transfected with vector alone.

Protein Expression and Purification

To express full-length twinfilin and each of its two cofilin-like repeats (amino acids 1–162 and 163–332, respectively) separately in *Escherichia coli*, appropriate fragments of the *TWF1* coding region were amplified from *S. cerevisiae* genomic DNA by PCR using oligonucleotides that introduce NcoI and HindIII sites at the 5′ and 3′ ends of the fragments, respectively. In full-length twinfilin and repeat-1 constructs, the 5′ oligonucleotide results in substitution of Ala for Ser at position 2. The PCR-fragments were ligated into NcoI-HindIII–digested pGAT2 plasmid (Peränen et al., 1996). All constructs were sequenced by the chain-termination method and the clones containing undesired mutations were discarded. Full-length twinfilin and each of its cofilin-like repeats were expressed as glutathione-*S*-transferase fusion proteins in *E. coli* BL21(DE3) cells under control of the P_{lac} promoter. Cells were grown in 2,000 ml of Luria broth to an optical density of 0.5 at 600 nm and then the expression was induced with 0.2 mM isopropyl-thio-β-D-galactoside (IPTG). Cells were harvested 3 h after induction, washed with 50 ml of 20 mM Tris (pH 7.5) and resuspended in 10 ml of PBS and 0.2 PMSF. Cells were lysed by sonication followed by a centrifugation for 15 min at 14,000 *g*. GST–fusion proteins were enriched from the supernatant using glutathione-agarose beads as described by Ausubel et al. (1990). GST–fusion proteins were incubated overnight at 4°C with thrombin (5 U/ml) to cleave twinfilin proteins away from GST. The beads were washed 3× with 50 mM Tris (pH 7.5) and 150 mM NaCl and the supernatants were concentrated to ~1.5 ml using Centricon 10-kD cutoff devices. Concentrated supernatants were loaded onto a Superdex-75

Table I. Yeast Strains Used in This Study

Strain	Genotype
DDY322	MAT α, *his3Δ200*, *leu2-3,112*, *ura3-52*, *Δabp1::LEU2*
DDY757	MAT a, *cry1*, *ade2-101*, *his3-11*, *leu2-3,112*, *ura3-1*
DDY759	MAT a/MAT α, *cry1/cry1*, *ade2-101/ade2-101*, *his3-11/his3-11*, *leu2-3,112/leu2-3,112*, *ura3-1/ura3-1*
DDY952	MAT α, *his3Δ200*, *leu2-3,112*, *ura3-52*, *lys2-801*, *srv2Δ2::HIS3*
DDY1024	MAT a, *ura3-52*, *lys2-801*, *his3Δ200*, *leu2-3,112*, *ade 2-101*, *ade3*, *pfy1-116:LEU2*
DDY1102	MAT a/MAT α, ade2-1/+, *his3Δ200/his3Δ200*, *leu2-3,112/leu2-3,112*, *ura3-52/ura3-52*, *lys2-801/+*
DDY1254	MAT α, *ura3-52*, *his3Δ200*, *leu2-3,112*, *lys2-801*, *cof1-5::LEU2*
DDY1266	MAT α, *ura3-52*, *his3Δ200*, *leu2-3,112*, *lys2-801*, *cof1-22::LEU2*
DDY1434	MAT a, *ade2-1*, *his3Δ200*, *leu2-3,112*, *ura3-52*, *Δtwf1::URA3*
DDY1435	MAT α, *ade2-1*, *his3Δ200*, *leu2-3,112*, *ura3-52*, *Δtwf1::URA3*
DDY1436	MAT a/MAT α, *ade2-1/ade2-1*, *his3Δ200/his3Δ200*, *leu2-3,112/leu2-3,112*, *ura3-52/ura3-52*, *Δtwf1::URA3/Δtwf1::URA3*
DDY1437	MAT a/MAT α, *ade2-1/+*, *his3Δ200/his3Δ200*, *leu2-3*, *112/leu2-3,112*, *ura3-52/ura3-52*, *lys2-801/+*, *Δtwf1:URA3/+*

HiLoad gel-filtration column (Pharmacia Biotech, Inc., Piscataway, NJ) equilibrated with 10 mM Tris (pH 7.5), 50 mM NaCl to remove thrombin and further purify the twinfilin proteins. The peak-fractions containing twinfilin and repeat-1 (eluted from the column at 56 and 67 ml, respectively) were pooled, concentrated in Centricon 10-kD cutoff devices to a final concentration of 30–100 μM, frozen in liquid N_2, and then stored at −80°C. The full-length twinfilin and repeat-1 were >90% pure, based on Coomassie-stained SDS–polyacrylamide gels. Yeast actin and yeast cofilin were purified as described previously by Lappalainen et al. (1997).

Actin Cosedimentation Assays

For the first set of F-actin cosedimentation assays (see Fig. 2), 40-μl aliquots of 0/2.5/5/7.5/10 μM yeast actin were polymerized for 45 min in F-buffer (10 mM Tris, pH 7.5, 0.7 mM ATP, 0.2 mM $CaCl_2$, 100 mM KCl, 0.2 mM DTT, and 2 mM $MgCl_2$). After polymerization, 10 μl of 10 μM twinfilin or repeat-1 (in 10 mM Tris, pH 7.5, and 50 mM NaCl) were mixed with actin and incubated at room-temperature for 15 min. For the second set of cosedimentation assays (see Fig. 3), 40-μl aliquots of 2.5 μM yeast actin were polymerized in F-buffer for 45 min, mixed with 10 μl of 0, 2.5, 5, 10, 20, 30, and 40 μM purified twinfilin or repeat-1, and incubated at room temperature for 15 min. The actin filaments were sedimented by centrifugation at 90,000 rpm for 20 min at 20°C in a TLA-100 rotor (Beckman Instruments, Inc., Fullerton, CA), and equal portions of the pellets and the supernatants were loaded onto 10 or 12% SDS gels. The gels were Coomassie-stained and the intensity of twinfilin and actin bands was quantified using an IS-1000 densitometer (Alpha Innotech Corporation, San Leandro, CA).

Monomer Binding Assays

Interactions of twinfilin and repeat-1 with actin monomers were monitored by native-gel electrophoresis and by the inhibition of actin nucleotide exchange. For native gel electrophoresis, the twinfilin and yeast actin were mixed to a final concentration of 15 μM each and electrophoresis was carried out as described by Safer (1989). For nucleotide exchange assays, 40 μl of G-buffer (10 mM Tris, pH 7.5, 2 mM $CaCl_2$, 2 mM DTT, and 25 mM ATP) containing 2.5 μM yeast actin and 0, 2, and 4 μM twinfilin or repeat-1 were mixed with 10 μl of 1 mM etheno-ATP. The nucleotide exchange reaction was followed at 20°C in a F-4010 fluorescence spectrophotometer (Hitachi Instruments, Inc., San Jose, CA) at an excitation of 360 nm and emission of 410 nm.

Depolymerization Assay

Kinetics of actin filament disassembly were monitored by pyrene fluorescence with excitation at 365 nm and emission at 407 nm. 6 μM actin (5:1 ratio of yeast actin/pyrene-labeled rabbit skeletal muscle actin) was polymerized in F-buffer for 45 min in the presence of 5 nM human platelet gelsolin. Depolymerization of the F-actin was induced by mixing 40 μl of the F-actin with 10 μl of 250 μM latrunculin-A, 30 μM Twf1, 30 μM yeast cofilin, or 10 μM yeast cofilin plus 250 μM latrunculin-A, and monitored for 10 min by the decrease in fluorescence at 407 nm in the fluorescence spectrophotometer.

In Vitro Kinase Assay

Purified full-length twinfilin (80 ng) was mixed with 5 μg of myelin basic protein (Sigma Chemical Co., St. Louis, MO) or poly(Glu-Tyr) 4:1 (Sigma Chemical Co.) in 30 μl of kinase reaction buffer (50 mM Hepes, pH 7.5, 60 mM KAc, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM ATP). 5 μCi of γ-[^{32}P]ATP (Amersham Corp., Arlington Heights, IL) was added on each reaction. After incubation for 15 min at room temperature, 10 μl of 4× SDS gel sample buffer (Laemmli, 1970) was added, and the proteins were resolved on a 10% SDS–polyacrylamide gel. ^{32}P-labeled bands were visualized by autoradiography after exposure of dried gels to X-ray film at −70°C.

Miscellaneous

PAGE in the presence of sodium dodecyl sulfate was carried out using the buffer system of Laemmli (1970). The concentrations of twinfilin and repeat-1 were determined by comparison to standard curves of known amounts of purified yeast cofilin and actin on Coomassie-stained SDS–polyacrylamide gels. Protein concentrations were determined using this method, rather than the calculated extinction coefficient, to avoid the possible contributions from small amounts of contaminating protein to absorbance at 280 nm.

Results

Identification of a Novel Actin-binding Protein in Yeast

In a search of the yeast *Saccharomyces cerevisiae* genome database for proteins with sequence homology to the actin filament depolymerizing protein cofilin, we discovered a previously uncharacterized open reading frame (YGR080W) encoding a novel cofilin-like protein. This gene, predicted to encode a 332–amino acid protein, is composed of not one, but two cofilin-like sequences with 21% and 19% amino acid sequence identity to yeast cofilin (Lappalainen et al., 1998). These cofilin-like repeats also show ~15% sequence identity to each other, and therefore we named this protein twinfilin (*TWF1*). The protein encoded by *TWF1* also shows homology to the human and mouse A6 proteins, previously identified by a screen of an embryonic cDNA expression library using a anti-phospho-tyrosine antibody (Beeler et al., 1994) and to a sequence in the *C. elegans* genome (these data are available from GenBank/EMBL/DDBJ under accession number U46668). Biochemical analyses in this earlier report suggested that human A6 protein might be a protein tyrosine kinase. However, A6 protein lacks any sequence homology to known protein kinases. Furthermore, we did not detect any kinase activity for purified yeast twinfilin using identical substrates and conditions to those described by Beeler et al. (1994; data not shown; see Materials and Methods).

A sequence alignment of yeast cofilin and twinfilin repeats 1 and 2 from yeast, human, and mouse (Fig. 1) shows that the positions of cofilin secondary structure elements identified from the yeast cofilin crystal structure (Fedorov et al., 1997) are relatively well conserved between cofilin and twinfilins. Furthermore, the sequence insertions in the twinfilins are located in regions predicted to form loops, suggesting that each twinfilin repeat has a tertiary structure similar to the ADF/cofilin proteins. Each twinfilin repeat also has an ~20–amino acid extension at its COOH-terminal region not found in any of the known ADF/cofilin proteins (Lappalainen et al., 1998).

The residues of cofilin that have been shown to be essential for interactions with actin monomers and actin filaments (indicated by asterisks above the sequences in Fig. 1) also are relatively well conserved in each repeat of twinfilins (see Fig. 1). However, the residues in yeast cofilin that have been shown to be essential for binding to actin filaments are less conserved in twinfilins than residues implicated in monomer binding. The overall structural conservation as well as the conservation of the actin-binding residues between cofilin and twinfilins suggests that twinfilins might bind directly to actin, and that their interactions with actin may be similar to ADF/cofilin proteins. In support of the hypothesis that twinfilin is an actin-binding protein, we also have identified twinfilin as a protein enriched from yeast extracts on an actin affinity column (Goode, B.L., D. Shieltz, J. Yates, and D.G. Drubin, unpublished observations).

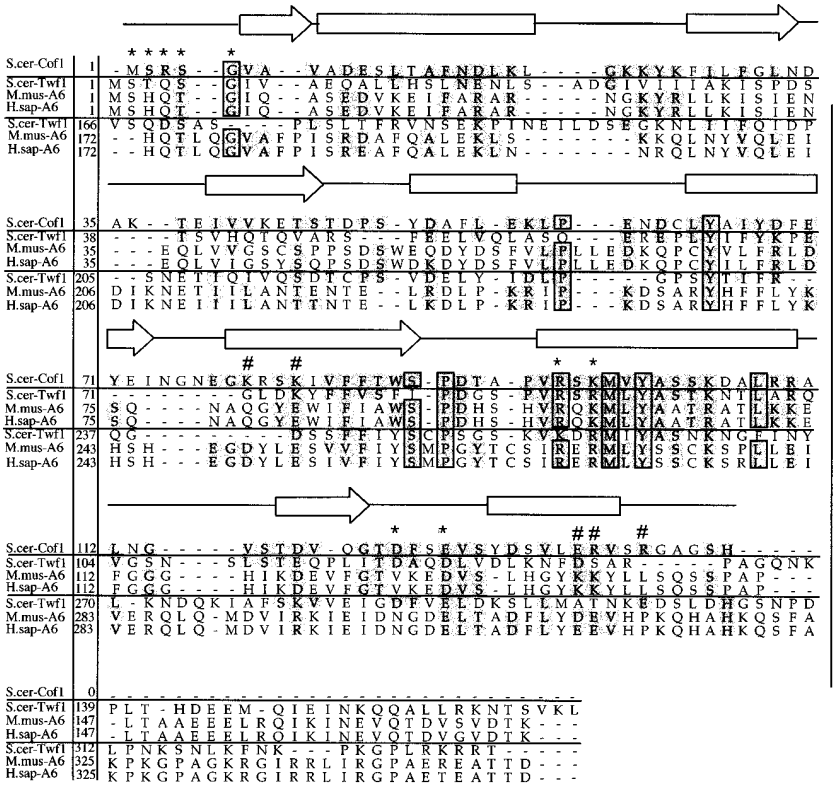

Figure 1. Sequence alignment of yeast cofilin and repeats 1 and 2 of yeast, mouse and human twinfilins. The secondary structure elements identified from the yeast cofilin crystal structure are shown above the sequences. The twinfilin sequences that are either identical or >85% conserved with yeast cofilin are highlighted and boxed, respectively. Yeast cofilin sequences that have been shown to be essential for interactions with actin also are marked (*, G/F-actin binding; #, F-actin binding). Note that COOH-terminal extensions not found in cofilin share homology among twinfilins.

Twinfilin Sequesters Actin by Forming a 1:1 Complex with Actin Monomers

To test directly whether twinfilin binds to actin filaments and/or actin monomers, we expressed full-length twinfilin in *E. coli* as a glutathione-*S*-transferase fusion protein. The twinfilin GST–fusion proteins were purified from *E.coli* extracts using glutathione-agarose beads. Twinfilin was subsequently cleaved from GST by digestion with thrombin. Purification was finalized by gel-filtration chromatography on a Superdex-75 column. The majority (70–80%) of the full-length twinfilin eluted from the column as a single peak at the expected position for a monomer (at ~ 54 ml). However, a small fraction of twinfilin (20–25%) eluted in the void volume, suggesting some aggregation under these conditions. After freezing and thawing of the monomeric fraction of twinfilin, we again observed that 20–25% of the twinfilin sedimented on its own upon ultracentrifugation for 20 min at 90,000 rpm in a TLA-100 rotor (Fig. 2, lane *2*). In the pelleting assays described below, the presence of the insoluble twinfilin fraction that pellets on its own is subtracted from the results. In monomer binding gel shift assays (see Fig. 4), the insoluble twinfilin fraction appears to not enter the gel and therefore should not effect on the results.

To study the interactions of the purified twinfilin with actin filaments, we first carried out actin filament cosedimentation assays using a constant concentration (2 μM) of twinfilin and variable concentrations (0–8 μM) of purified yeast F-actin. As shown in Fig. 2, addition of twinfilin to the actin filaments leads to a significant increase in the amount of actin present in the supernatant. Whereas ~90% of the actin is normally found in the pellet fraction under these conditions, addition of equimolar amounts of twinfilin to F-actin decreases the amount of actin in the pellet to ~25% (compare Fig. 2, lane *1* with *3*). At higher actin concentrations twinfilin appears to shift actin to the supernatant in an ~1:1 molar ratio (i.e., for every molecule of twinfilin added to the reaction, one molecule of actin shifts to the supernatant). Several different mechanisms of action by twinfilin could underlie these observations, including actin monomer sequestration and/or capping of the barbed end of the filaments to prevent new subunit addition; however, the 1:1 stoichiometry of actin and twinfilin in the supernatant strongly suggests monomer sequestration. Fig. 2 also shows that only a small increase in the amount of twinfilin in the pellet occurs upon addition of increasing concentrations of F-actin, suggesting that twinfilin does not bind tightly to actin filaments.

To further examine the ability of twinfilin to depolymerize actin filaments, we carried out a cosedimentation assay

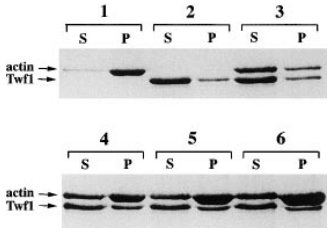

Figure 2. Twinfilin–actin sedimentation assay. This assay was performed by mixing 2 μM twinfilin with 0 μM (lanes *1*), 2 μM (lanes *3*), 4 μM (lanes *4*), 6 μM (lanes *5*), or 8 μM (lanes *6*) prepolymerized actin filaments. In the absence of twinfilin ~90% of actin pelleted (lane *1*, *P* = pellet, S = supernatant). Upon addition of 2 μM twinfilin, the amount of actin in the supernatant increased (see lanes 3–6). Approximately 20–25% of twinfilin sedimented on its own (lane *2*), and there was only a modest increase in the amount of twinfilin in the pellet at actin concentrations between 0 and 8 μM, indicating that twinfilin does not interact tightly with actin filaments.

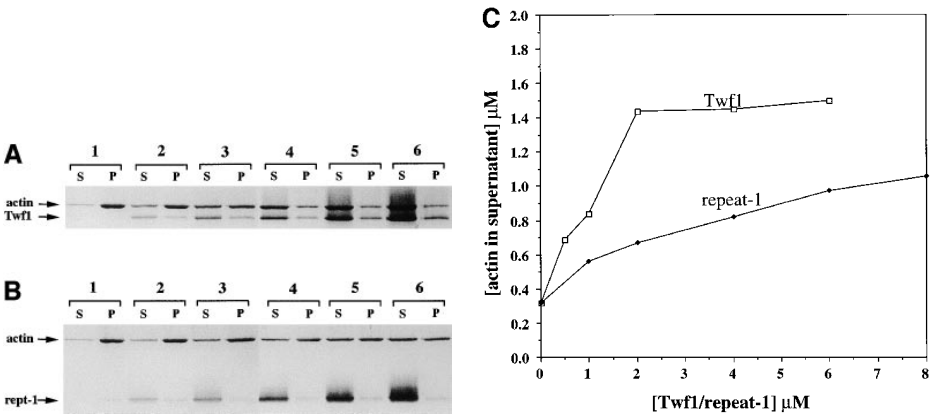

Figure 3. Twinfilin and its first cofilin homology domain each decrease the amount of actin filament in solution. (*A*) 2 μM yeast actin was polymerized for 40 min and mixed with 0 μM (lane *1*), 0.5 μM (lane *2*), 1 μM (lane *3*), 2 μM (lane *4*), 4 μM (lane *5*), or 6 μM (lane *6*) twinfilin. Twinfilin increases the amount of actin in the supernatant and decreases the amount of actin in the pellet. (*B*) 2 μM yeast actin was polymerized and mixed with 0 μM (lane *1*), 1 μM (lane *2*), 2 μM (lane *3*), 4 μM (lane *4*), 6 μM (lane *5*), or 8 μM (lane *6*) of twinfilin repeat-1. The repeat-1 increases the amount of actin in the supernatant in a concentration dependent manner, but less efficiently than full-length twinfilin. (*C*) Quantification of the amount of actin in the supernatant (y axis) with various concentrations of twinfilin and repeat-1 (x axis). Because ~20–25% of twinfilin sediments on its own, and because the binding of twinfilin to actin monomers is essentially saturated at 2 μM yeast actin (see panel *A*.), these data are consistent with formation of a 1:1 complex between twinfilin and actin monomers.

using a constant (2 μM) concentration of actin and variable twinfilin concentrations (0 and 6 μM). As shown in Fig. 3, *a* and *c*, addition of twinfilin causes an equimolar amount of actin to redistribute from the pellet fraction to the supernatant. Because ~25% of twinfilin pellets on its own, the addition of 2 μM twinfilin to actin filaments can shift a maximum of 1.5 μM actin to the supernatant. The observation that twinfilin–actin monomer binding saturates at 2 μM twinfilin and 2 μM actin (see Fig. 2) strongly suggests that twinfilin forms a tight 1:1 complex with actin monomers.

To further test the ability of twinfilin to form a complex with actin monomers, we used native gel electrophoresis. Reactions containing a final concentration of 15 μM twinfilin and 15 μM yeast actin in G-buffer (see Materials and Methods) were fractionated on a 7.5% native polyacrylamide gel run at 100 V for 120 min. The motilities of yeast actin and recombinant twinfilin alone are shown in Fig. 4 (lanes *1* and *2*, respectively). Lane *3* in Fig. 4 shows that addition of twinfilin to actin at a 1:1 molar ratio causes in a dramatic shift in the motility of actin, resulting in the formation of a smear between the twinfilin and actin bands. These results suggest that the complex that forms between twinfilin and actin is relatively stable in vitro. As an independent test of monomer binding, we also investigated whether addition of twinfilin could inhibit nucleotide exchange that normally occurs for actin monomers in solution. Inhibition of nucleotide exchange has been demonstrated previously for ADF/cofilin proteins (Nishida, 1985). As shown in Fig. 5, twinfilin inhibits nucleotide exchange of ATP-actin monomers in a concentration-dependent manner similar to that described for cofilin (Nishida, 1985). Taken together, these data (the shift in F-actin steady state towards monomer in a 1:1 molar ratio, the detection of twinfilin/actin monomer complexes on native gels, and the inhibition of nucleotide exchange by actin when twinfilin is bound) demonstrate that twinfilin functions in vitro like a bona fide actin monomer-sequestering protein.

ADF/cofilin proteins have been shown to promote the rapid depolymerization of actin filaments from their pointed ends (Carlier et al., 1997). Because twinfilin has sequence homology to ADF/cofilin proteins, we tested whether it also effects actin filament depolymerization kinetics in a similar manner to ADF/cofilin proteins. To specifically measure the filament depolymerization from the pointed end, actin filaments capped at their barbed ends were prepared by polymerizing 6 μM yeast actin in the presence of 5 nM gelsolin as described by Carlier et al. (1997). Depolymerization was then induced by the addition of twinfilin, cofilin or the actin monomer-sequestering drug latrunculin-A. As shown in Fig. 6, twinfilin and latrunculin-A have similar effects in this assay, consistent with the conclusion that twinfilin sequesters actin monomers without stimulating filament depolymerization. In contrast, cofilin stimulates rapid filament depolymerization. Because the stoichiometric cofilin-induced depolymerization was too rapid to monitor using our experimental system (Fig. 6 *D*), we also performed this assay using substoichiometric cofilin and a sufficient concentration of latrunculin A to sequester depolymerized actin monomers (Fig. 6 *C*). These conditions allowed detection of the cofilin-induced rapid actin filament depolymerization. It is important to note that the rapid decrease in signal may be in part due to fluorescence quenching of the pyrene actin upon cofilin binding (Carlier et al., 1997). However, it is clear from our results that, despite its sequence homology with ADF/cofilin proteins, twinfilin has little or no effect on filament depolymeriza-

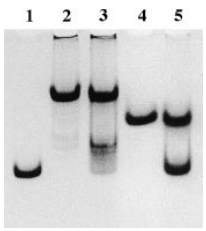

Figure 4. Analysis of twinfilin–actin monomer complexes on native gels. Lane *1*, actin alone; lane *2*, twinfilin; lane *3*, actin + twinfilin; lane *4*, repeat-1; and lane *5*, repeat-1 + actin. Each protein was mixed in solution and then loaded on the gel at a final concentration of 15 μM. Addition of twinfilin to actin (lane *3*) causes in a shift in the motility of actin and formation of a smear between the actin and twinfilin bands. This result is consistent with the formation of a high affinity complex between twinfilin and actin monomers. Mixing of actin 1:1 with repeat-1 results in some smearing between actin and repeat-1 bands (lane *5*), indicating formation of a lower affinity complex between these two proteins.

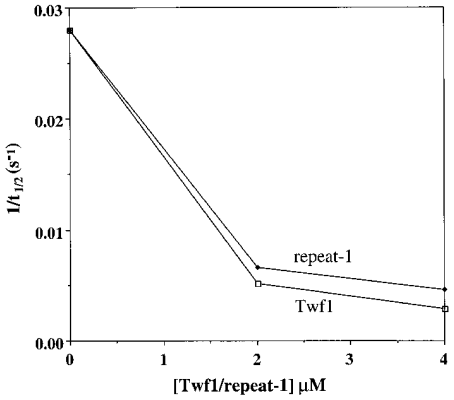

Figure 5. Effects of twinfilin and repeat-1 on the nucleotide exchange of yeast actin monomers. The reaction-rates are indicated on the y axis as the inverse of the reaction half-life ($t_{1/2}$). Both full-length twinfilin and repeat-1 inhibit the nucleotide exchange in a concentration-dependent manner.The final concentration of actin in these reactions was 2 μM.

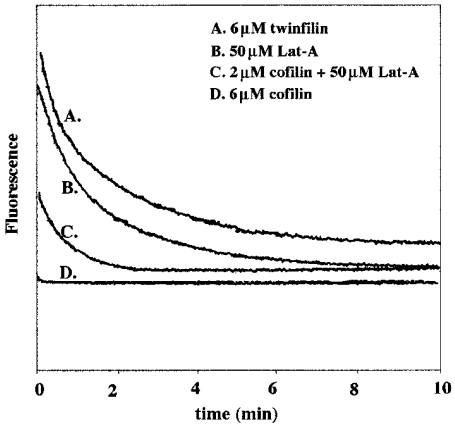

Figure 6. Depolymerization assay using yeast actin filaments capped at their barbed-ends with gelsolin. Actin filaments (6 μM, 1:5 pyrene rabbit actin/yeast actin) were polymerized in the presence of 5 nM gelsolin. Depolymerization was induced by mixing 40 μl of actin with 10 μl of twinfilin (*A*), latrunculin-A (*B*) or cofilin (*C* and *D*). The depolymerization of actin filaments was followed by the decrease in the fluorescence at 407 nm. Addition of twinfilin and the monomer-sequestering drug latrunculin-A each results in a slow depolymerization of actin filaments relative to the rapid actin filament depolymerization induced by cofilin. 2 μM cofilin with 50 μM monomer-sequestering drug latrunculin-A (*C*) causes a significantly faster filament depolymerization than 6 μM twinfilin. The filament depolymerization induced by 6 μM cofilin is extremely rapid (*D*) and is completed before a measurement can be made (20–30 s).

tion rate at the pointed end. One potential caveat of these experiments is that the recombinant twinfilin used in these assays could be improperly folded. However, the recombinant twinfilin eluted as a single peak on a gel filtration column and showed strong activities on actin, suggesting that these preparations are homogeneous and active. On the other hand, these results do not rule out the possibility that native yeast twinfilin might have additional activities not detected here for the recombinant protein.

Both Cofilin-like Repeats in Twinfilin Are Required for Strong Actin Monomer Binding

Because twinfilin is composed of two ADF homology domains (repeats 1 and 2), we investigated whether individual repeat domains might have activities similar to those of full-length twinfilin. Each repeat was expressed in *E. coli* as a GST–fusion protein and purified as described above for full-length twinfilin. As shown in Table II, both repeats were expressed at high levels in *E. coli*, but only repeat-1 was soluble after cleavage from GST with thrombin. All of the repeat-2 eluted in void volume from the Superdex-75 gel-filtration column after cleavage from glutathione-*S*-transferase and sedimented on its own upon ultracentrifugation for 20 min at 90,000 rpm in TLA-100 rotor. Therefore, only repeat-1 was used for the assays described below.

Table II. Bacterial Expression and Solubility of Yeast Twinfilin and Its Two Cofilin Homology Domains Expressed Individually

	Expression	Solubility
1 repeat-1 repeat-2 332	+	+
1 repeat-1 162	+++	+++
163 repeat-2 332	+++	−

In cosedimentation assays using a range of F-actin concentrations (0–8 μM) and a constant repeat-1 concentration (2 μM), repeat-1 did not exhibit copelleting with actin filaments (data not shown). However, repeat-1 was able to increase the amount of actin in the supernatant in a concentration-dependent manner (Fig. 3, *b* and *c*). This activity was significantly weaker than that observed for full-length twinfilin, which suggests that both repeats are necessary for strong actin monomer sequestering. We also tested directly the interaction of repeat-1 with actin monomers by native gel electrophoresis and by the inhibition of nucleotide exchange on actin monomers. As shown in Fig. 4 (lanes *4* and *5*) addition of repeat-1 to G-actin results in the formation of a weak smear between the repeat-1 and the actin bands, suggesting that these two proteins form a low affinity molecular complex with each other. As shown in Fig. 5, Repeat-1 also inhibits nucleotide exchange by actin monomers in a concentration dependent manner. Together, these results suggest that repeat-1 forms a complex with actin monomers similar in nature, but weaker in strength to the one formed by cofilin and full-length twinfilin.

A GFP–Twinfilin Fusion Protein Localizes to the Cytoplasm and the Cortical Actin Cytoskeleton in Yeast Cells

To investigate the localization of twinfilin protein in yeast, a GFP–twinfilin fusion protein was expressed and its local-

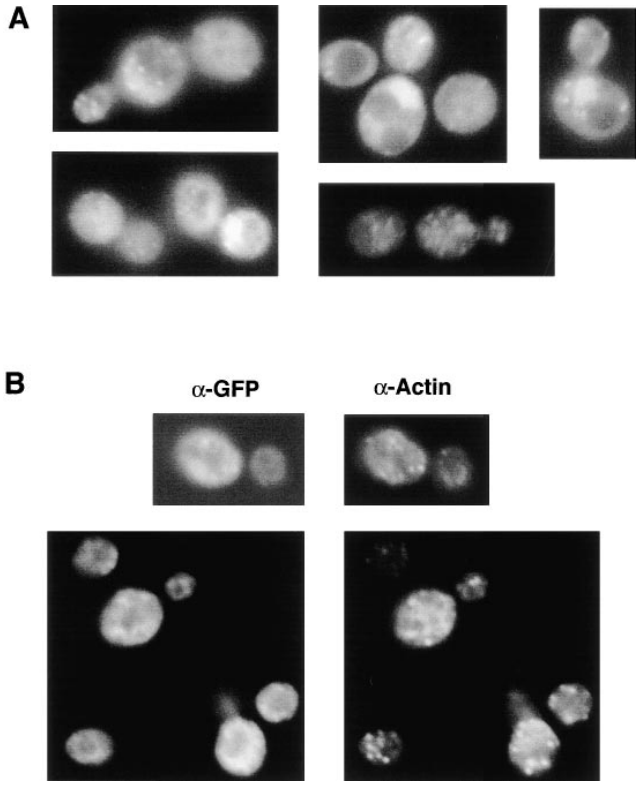

Figure 7. Localization of GFP–twinfilin fusion protein in yeast cells. DDY759 cells were transformed with a plasmid encoding a GFP–twinfilin fusion protein under regulation of the galactose promoter. To induce expression of the GFP fusion protein, cells were grown in synthetic glucose medium lacking uracil, harvested at log phase by centrifugation, and switched to galactose medium lacking uracil for 12 h. Living cells were then mounted and visualized by light microscopy. (*A*) Cytoplasmic and patch-like staining patterns of GFP–twinfilin observed in living cells. (*B*) Double immunofluorescence labeling of cells expressing the GFP–twinfilin fusion protein using anti-actin and anti-GFP antibodies. The GFP–twinfilin patch staining overlaps with a subset of cortical actin patches.

ization observed in living cells. As shown in Fig. 7 *a*, the majority of cells expressing the GFP–twinfilin fusion protein showed strong cytoplasmic staining with additional cortical punctate staining. The cortical spot structures moved in real time, with some of the patches holding a stable position and others dramatically translocating over the span of seconds (data not shown). Since these movements are very similar to those of cortical actin patches described in previous reports (Doyle and Botstein, 1996; Waddle et al., 1996), we used double immunofluorescence with anti-actin and anti-GFP antibodies to address whether the GFP–twinfilin patches correspond to actin patches. In the majority of cells examined, the anti-GFP staining localized primarily to the cytoplasm, but many cells also showed patch-like staining. Examples of cells with clear patch staining are shown in Fig. 7 *b*. In these cells, the GFP-staining patches overlapped with a subset of the cortical actin patches. Taken together, these results suggest that twinfilin localizes primarily to the cytoplasm, but also to the cortical actin cytoskeleton. However, it is important to remember that this localization was carried out in cell overexpressing GFP–twinfilin fusion protein and may therefore not fully represent the localization of twinfilin in wild-type cells. While the cytoplasmic localization of twinfilin is consistent with its activities as an actin monomer-sequestering protein, the patch-like staining raises intriguing possibilities about the regulation of twinfilin function. One possibility is that a fraction of twinfilin is associated with cortical actin patches through binding interactions with patch components other than actin. This also could explain the above mentioned isolation of twinfilin from yeast extracts on actin filament affinity columns.

Deletion of the TWF1 Gene Results in Synthetic Lethality with a Cofilin Mutant

To investigate the in vivo functions of twinfilin, we generated a strain in which the *TWF1* gene is deleted and replaced by the *URA3* gene. Haploid *twf1Δ* cells exhibit normal growth and have normal morphologies over a temperature range of 20–37°C (data not shown). The growth of *twf1Δ* cells also was indistinguishable from wild-type cells on a variety of stressful media, including media produced with low and high pH, high NaCl, KCl, $MgCl_2$, $CaCl_2$, and formamide (data not shown). Furthermore, *twf1Δ* cells have no detectable defects in fluid phase endocytosis (data not shown; see Materials and Methods) and their actin cytoskeletons appear normal by immunofluorescence except for consistently brighter actin patch staining (Fig. 8).

The absence of a strong detectable phenotype in *twf1Δ* cells suggests that there may be functional redundancy be-

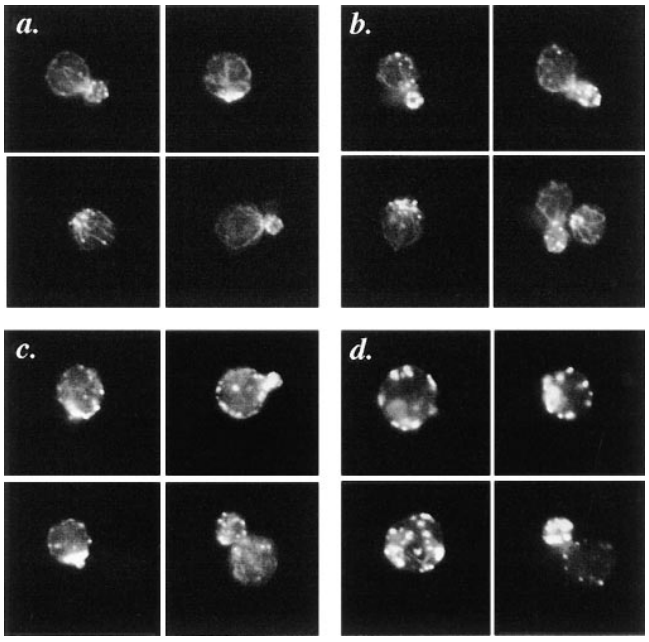

Figure 8. Organization of the actin cytoskeleton in wild-type (*A*), *twf1Δ* (*B*), *cof1-22* (*C*) and *twf1Δ* × *cof1-22* (*D*) cells. The cells were grown at 20°C to an OD_{600} of ~0.3, fixed with formaldehyde, and then the actin was visualized by immunofluorescence. The *twf1Δ* cells appear to have slightly brighter cortical actin patches than the wild-type cells, whereas the cortical actin structures in *cof1-22* cells are significantly brighter than in wild-type cells. The actin patches in *twf1Δ* × *cof1-22* double mutant are unusually large, irregularly shaped, and mostly depolarized.

tween twinfilin and other proteins in yeast. Because the actin cytoskeleton is characterized by a high complexity of protein components, and by many examples of genetic redundancy, gene disruption of one actin binding protein often has no significant effects on growth rate of cells or the overall appearance of the actin cytoskeleton by immunofluorescence. However, such gene disruptions can lead to strong synergistic defects in combination with other mutations in genes that encode actin-binding proteins (e.g., Holtzman et al., 1993). To test the possibility that functional redundancy explains the lack of pronounced defects in *twf1Δ* mutants, we crossed *twf1Δ* mutants with mutants of other genes encoding actin binding proteins, concentrating on genes that encode proteins with ADF-homology domains (*COF1* and *ABP1*; Lappalainen et al., 1998) and on genes that encode known actin monomer binding proteins (profilin/*PFY1*, cofilin/*COF1*, and *SRV2*). As shown in Table III, *twf1Δ* demonstrates a strong and specific synthetic phenotype with the cofilin allele *cof1-22*. This cofilin mutant has been shown to have significant defects in F-actin binding and depolymerization both in vivo and in vitro, and it results in lethality at the temperatures >30°C. However, at 20°C *cof1-22* cells show normal morphology and exhibit growth rates similar to wild-type cells (Lappalainen and Drubin, 1997). After 3 d at 20°C, none of the *twf1Δ cof1-22* double mutants formed visible colonies. However, after prolonged incubation (5–7 d at 20°C), tiny *twf1Δ cof1-22* colonies appeared. The cells in these colonies were abnormally large. To visualize the actin cytoskeletons in such *twf1Δ cof1-22* cells, the segregants were inoculated into a small volume of YPD and grown at 20°C for 48 h. Fig. 8 shows a comparison of the morphologies of the actin cytoskeletons in wild-type, *twf1Δ* cells, *cof1-22*, and *twf1Δ cof1-22* double mutant cells grown at 20°C. Whereas *twf1Δ* and *cof1-22* cells show some increase in the brightness (= size) of the cortical actin structures compared with wild-type cells, most *twf1Δ cof1-22* double mutant cells have completely depolarized cortical actin cytoskeletons and abnormally large and chunky actin patches. These results suggest that *TWF1* and *COF1* genes may share a function required for the regulation of actin-based processes.

Deletion of the TWF1 Gene Causes Random Budding Pattern and Bumpy Surface Morphology in Diploid Yeast Cells

We also examined the morphology of diploid yeast cells homozygous for the *twf1Δ* gene deletion (DDY1436). Fig. 9 *a* shows that *twf1Δ/twf1Δ* cells appear to form normal buds, but the cells have large bumps on their surfaces. Similar phenotypes have been reported previously for a subset of actin alleles that have defects in bipolar bud patterning (Drubin et al., 1993; Yang et al., 1997). Calcofluor staining of the *twf1Δ/twf1Δ* cells revealed that each bump is marked by a bud scar, suggesting that the bumps represent sites of past bud formation and cytokinesis. In normal diploid yeast, the first bud to emerge from a daughter cell is usually formed at the pole opposite to the birth scar, and subsequent buds form at sites that are either at the same pole as the birth scar or the opposite pole (Chant and Pringle, 1995). In wild-type cells, this leads to a bipolar budding pattern (the accumulation of multiple bud scars positioned at either pole). It has been shown that disruption of the actin cytoskeleton does not affect the position of the first bud to emerge from the daughter cell, but subsequently results in a random budding pattern in diploid cells (Yang et al., 1997). Diploid-specific bud pattern defects also have been observed in actin-binding protein mutants, including *sla2Δ*, *rvs167Δ*, and *sac6Δ* (Drubin et al., 1993; Yang et al., 1997). We found that 56% of *twf1Δ/twf1Δ* cells exhibit random bud scar patterning (examples are shown in Fig. 9 *b*), compared with only 2% of wild-type cells. Such frequencies of random budding are similar to those reported previously for actin and actin-binding protein mutants, and support the model that *TWF1* is involved in actin cytoskeletal functions in vivo.

Table III. Genetic Interactions between Δtwf1 and Actin-binding Protein Mutants

	Double mutants forming colonies over the total number of double mutants
Δtwf1 × pfy1-116	5/5
Δtwf1 × cof1-5	11/11
Δtwf1 × cof1-22	0/9
Δtwf1 × Δsrv2	7/7
Δtwf1 × Δabp1	9/9

Double mutants were inferred by marker segregation and colonies were scored three days after tetrad dissection at 20°C.

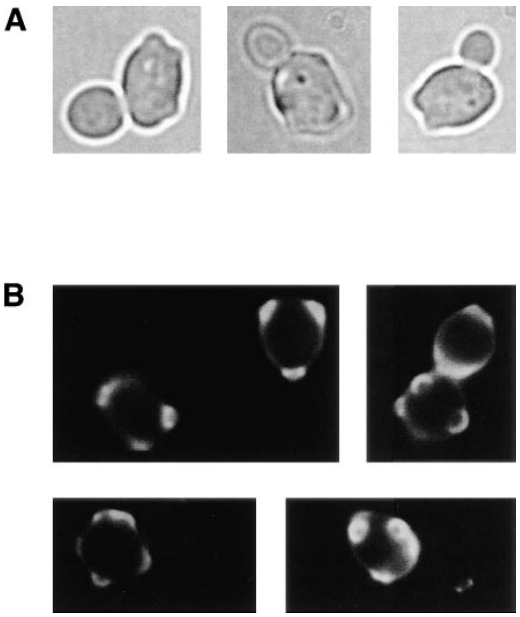

Figure 9. Budding pattern defects and bumpy surface abnormalities in *twf1Δ/twf1Δ* cells. (*A*) DIC imaging of living *twf1Δ/twf1Δ* cells reveals abnormal bumpy surface projections not found in wild-type diploid cells. (*B*) Calcofluor staining reveals a random bud scar pattern in 56% of *twf1Δ/twf1Δ* cells, as compared with only 2% random budding in wild-type cells.

Overexpression of Twinfilin Causes Depolarization of the Cortical Actin Cytoskeleton

Finally, we examined the effects of overexpressing twinfilin in yeast cells. One might predict that increased levels of

an actin monomer-sequestering protein would lead to reduced polymer levels, and the build up of a larger pool of sequestered actin monomers in the cytoplasm. Recently it has been shown that the overexpression of previously identified actin monomer-binding proteins in yeast have different effects on the actin cytoskeleton depending on the actin-binding protein (Hofmann, C., and D.G.Drubin, unpublished results). While overexpression of Srv2p has no detectable effects, overexpression of profilin and cofilin both lead to a partial depolarization of the cortical actin cytoskeleton and the formation of cytoplasmic actin bars (aberrant structures that are not likely to be composed of filamentous actin since they do not stain with rhodamine-phalloidin). As shown in Fig. 10, overexpression of twinfilin leads to a complete depolarization of the actin cytoskeleton and the accumulation of cytoplasmic actin bars.

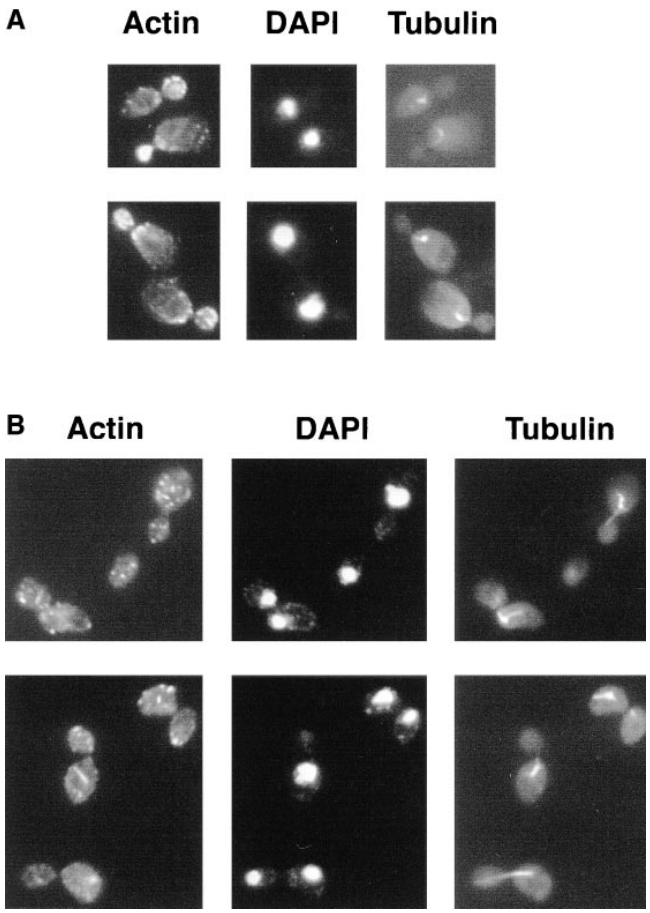

Figure 10. Effects of overexpression of twinfilin in yeast cells. Wild-type diploid cells (DDY759) were transformed with either a control plasmid (pRB1438) or a plasmid carrying the *TWF1* gene under the regulation of the galactose promoter (pGAL-*TWF1*). To induce expression of twinfilin, cells were grown to log phase in synthetic glucose medium lacking uracil, then harvested by centrifugation and switched to galactose medium lacking uracil. After 16 h of growth at 25 °C, cells were fixed and prepared for double immunofluorescence using actin and tubulin antibodies, as well as DAPI staining of DNA. (*A*) Control cells have a normal, polarized actin cytoskeleton and normal microtubule morphologies. (*B*) Cells overexpressing twinfilin show normal microtubule staining and cell shape, but severely depolarized cortical actin patches and the formation of aberrant cytoplasmic actin bars.

Both of these effects support the conclusion that twinfilin functions as an actin monomer-sequestering protein in vivo.

Discussion

Twinfilin Is a Novel and Widely Conserved Actin Monomer-binding Protein

In this study, we have identified and characterized a novel actin monomer-binding protein in budding yeast. This protein, which we named twinfilin, is composed of two cofilin-like regions and appears to be evolutionarily conserved. Our database searches identified genes in *C. elegans*, humans, and mice that encode homologous proteins (~20% identical). Because the ADF/cofilin-like repeats 1 and 2 of twinfilin are more closely related across species than they are to each other within a given species, we can predict that twinfilin in human, mouse, and yeast cells evolved from a common ancestral twinfilin and, therefore, twinfilin represents a single protein family (Lappalainen et al., 1998).

In vitro and in vivo data in this paper strongly suggest that twinfilin functions as an actin monomer-sequestering protein. Previous biochemical analyses of the human homologue of twinfilin (named A6) suggested a different function. Human twinfilin/A6 was originally identified as a phosphoprotein from an embryonic cDNA expression library, and studies using recombinant human twinfilin/A6 suggested that this protein might be a novel protein tyrosine kinase (Beeler et al., 1994). However, twinfilin/A6 lacks any of the sequence motifs found in protein kinases, and we have been unable to detect any kinase activity for recombinant yeast twinfilin using identical substrates and conditions to those reported by Beeler et al. (1994). On the other hand, our biochemical and genetic data support the conclusion that twinfilin is an actin monomer-sequestering protein.

So far, only three classes of actin monomer-binding proteins that are conserved across species as diverse as yeast and mammals have been identified. These proteins are Srv2/CAP, ADF/cofilin and profilin (Freeman et al., 1995, Carlier and Pantaloni, 1997). Although the biochemical properties of Srv2/CAP are relatively poorly understood, the activities of ADF/cofilin and profilin from a variety of organisms have been characterized extensively. Our data show that the activities of twinfilin are distinct from those of ADF/cofilin and profilin, indicating that twinfilin makes a unique contribution to the regulation of the cytoskeleton. Whereas ADF/cofilin interacts with both actin monomers and filaments and induces rapid dissociation of actin monomers from the pointed ends of filaments (Carlier et al., 1997), twinfilin interacts primarily with actin monomers and has no significant effect on dissociation of subunits from filament ends. Furthermore, twinfilin-bound actin monomers are blocked from reassembly into actin filaments, whereas ADF/cofilin-bound actin monomers are readily added at the barbed ends of actin filaments (Carlier et al., 1997). Thus, at the protein concentrations used in this study, both twinfilin and ADF/cofilin bind to actin monomers, but only twinfilin sequesters monomers. These activities of twinfilin contrast even more sharply

with those of profilins, which stimulate nucleotide exchange on actin (Perelroizen et al., 1995) and promote actin assembly at the barbed end (reviewed by Carlier and Pantaloni, 1997). Thus, amongst all of the actin-binding proteins widely conserved in eukaryotes, twinfilin is unique in its ability to sequester actin monomers.

Despite the functional differences between cofilin and twinfilin mentioned above, several lines of evidence suggest that cofilin and twinfilin may interact with actin monomers through a similar interface. First, the overall sequence homology between these proteins suggests that they may have similar tertiary structures. Second, the high conservation of the residues that have been shown to be essential for actin monomer-binding in yeast cofilin suggests that twinfilin and cofilin may have similar actin interactions (Lappalainen et al., 1997, see Fig. 1). Finally, the similarity in their inhibition of the nucleotide exchange reaction on actin suggests that twinfilin and cofilin may bind to actin monomers at a common interface. From the inhibition of nucleotide exchange activities of twinfilin (Fig. 5) and cofilin (Lappalainen et al., 1997), it also appears that twinfilin and ADF/cofilin may bind to ATP-actin monomers with similar affinities (<1 μM). To more thoroughly understand the role of twinfilin in regulating actin filament turnover, in the future it will be important to measure directly the affinity of twinfilin for ADP- and ATP-actin monomers.

What then accounts for the distinct activities of ADF/cofilin and twinfilin on actin? We speculate that the presence of two covalently attached ADF-homology domains could stabilize twinfilin/actin monomer complexes by decreasing the dissociation rate of actin monomers and/or by masking sites on the actin monomer surface that are required for subunit addition at the barbed end. Consistent with this model, we have shown that strong actin monomer-sequestering activity of twinfilin requires both of its ADF homology domains.

A Role for Twinfilin in Regulation of the Yeast Cortical Actin Cytoskeleton

Several lines of evidence resulting from in vivo analyses of twinfilin function suggest a role in regulating assembly of the actin cytoskeleton. Deletion of the *TWF1* gene leads to an increase in the intensity of cortical actin patch staining, suggesting that twinfilin acts to limit actin filament assembly in vivo. Moreover, overexpression of twinfilin causes depolarization of the actin cytoskeleton and formation of cytoplasmic actin bars, and diploids homozygous for the twinfilin null mutation show a random budding pattern. These are signature phenotypes for yeast with defects in the cortical actin cytoskeleton.

More specifically, twinfilin may be involved in regulating levels of free actin monomer in cells. The twinfilin deletion mutation shows a pronounced negative synergism when combined with the *cof1-22* allele of the yeast cofilin gene. This cofilin allele exhibits both diminished F-actin binding and defects in actin filament depolymerization in vivo and in vitro (Lappalainen and Drubin, 1997; Lappalainen et al., 1997). The cells carrying *cof1-22* have enlarged, irregularly shaped actin patches, suggesting that these cells may have elevated levels of filamentous actin (Lappalainen and Drubin, 1997). Cells carrying the *cof1-22* allele are predicted to have an unusually low actin monomer pool due to defects in actin filament depolymerization. We suggest that the synthetic phenotype observed between *twf1Δ* and *cof1-22* arises from a synergistic depletion of unassembled actin. Thus, through its monomer-sequestering activity, twinfilin may function with cofilin to maintain a pool of actin monomers available for assembly.

Given the strong monomer-sequestering activity displayed by twinfilin in vitro, it is likely that twinfilin activity is tightly regulated in vivo. Possible mechanisms for such regulation include phosphorylation of twinfilin, physical interactions between twinfilin and other proteins (possibly other actin-binding proteins), and regulation by association with phospholipids. Phosphorylation and PIP_2 binding both have been shown previously to regulate actin-related activities of ADF/cofilin proteins (for review se; Moon and Drubin, 1995), and multiple protein interactions have been implicated in the regulation of actin-binding proteins in yeast (Lila et al., 1997). The observation that a fraction of GFP–twinfilin localizes to the cortical actin supports the model that twinfilin may interact with other proteins associated with cortical actin filaments. Consistent with this hypothesis, we have also isolated twinfilin from yeast extracts on an F-actin affinity column (B.L. Goode, D. Shieltz, J. Yates, and D.G. Drubin, unpublished observations). In future experiments, it will be important to identify twinfilin-interacting proteins. Such proteins might account for the partial colocalization of twinfilin with cortical actin patches. In particular, we wish to understand how one or more of the above-mentioned regulatory mechanisms may stimulate the release of actin monomer from twinfilin in cells, providing a stimulus-responsive pool of actin monomers.

We thank Paul Janmey (Harvard University) for a generous gift of human platelet gelsolin.

This study was supported by grants from National Institutes of Health to B.L. Goode (GM-17715), American Cancer Society to D.G. Drubin (GM-42759) and Human Frontier Science Program to P. Lappalainen.

Received for publication 24 March 1998 and in revised form 24 June 1998.

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