The* Schizosaccharomyces pombe* cdc3+ Gene Encodes a Profilin Essential for Cytokinesis

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Abstract. The fission yeast *Schizosaccharomyces pombe* divides by medial fission and, like many higher eukaryotic cells, requires the function of an F-actin contractile ring for cytokinesis. In *S. pombe*, a class of *cdc*-mutants defective for cytokinesis, but not for DNA replication, mitosis, or septum synthesis, have been identified. In this paper, we present the characterization of one of these mutants, *cdc3-124*. Temperature shift experiments reveal that mutants in *cdc3* are incapable of forming an F-actin contractile ring. We have molecularly cloned *cdc3* and used the *cdc3+* genomic DNA to create a strain carrying a *cdc3-null* mutation by homologous recombination in vivo. Cells bearing a *cdc3-null* allele are inviable. They arrest the cell cycle at cytokinesis without forming a contractile ring. DNA sequence analysis of the *cdc3+* gene reveals that it encodes profilin, an actin-monomer-binding protein. In light of recent studies with profilins, we propose that Cdc3-profilin plays an essential role in cytokinesis by catalyzing the formation of the F-actin contractile ring. Consistent with this proposal are our observations that Cdc3-profilin localizes to the medial region of the cell where the F-actin contractile ring forms, and that it is essential for F-actin ring formation. Cells overproducing Cdc3-profilin become elongated, dumbbell shaped, and arrest at cytokinesis without any detectable F-actin staining. This effect of Cdc3-profilin overproduction is relieved by introduction of a multicopy plasmid carrying the actin encoding gene, *act1+*. We attribute these effects to potential sequestration of actin monomers by profilin, when present in excess.

The three major landmark events in the eukaryotic cell cycle are (a) replication of the genetic material that occurs in S phase; (b) partitioning of the replicated DNA to the daughter nuclei during M phase; and (c) division of the cell itself to produce two daughter cells as a result of cytokinesis. The last 10 yr have seen tremendous advances in our understanding of the molecular nature of processes occurring during the S and M phases and the controls that regulate entry into S and M phases. By contrast, the mechanisms and regulations that govern the third major landmark event, cytokinesis, remain only poorly understood.

Much of the progress in our understanding of the controls that regulate S and M phases stem from genetic studies in the fission yeast *Schizosaccharomyces pombe*. Several lines of evidence suggest that this yeast should also be an ideal system with which to study cytokinesis. First, *S. pombe* cells divide by medial fission (Nurse, 1985) and, like many higher eukaryotic cells, produce equally sized daughter cells after cytokinesis. Second, cytokinesis in fission yeast, akin to higher eukaryotic cells, uses the function of an F-actin contractile ring (Marks and Hyams, 1985; Jochoviči et al., 1991). Third, conditionally lethal mutants that are defective for cytokinesis have been isolated from screens designed to identify mutants defective for cell cycle progression. These mutants uncouple the nuclear events of the cycle from cytokinesis so that under nonpermissive conditions, they accumulate multiple nuclei but do not undergo cytokinesis (Nurse et al., 1976). Mutant defective for cytokinesis have been classified into two major categories, early cell plate mutants and late cell plate mutants. Under restrictive temperature conditions, early cell plate mutants, defined by genetic loci *cdc7, cdc11, cdc14*, and *cdc15*, arrest without detectable septum material after accumulating 8-16 nuclei (Nurse et al., 1976). Molecular and genetic analyses suggest that these genes might regulate some early aspect of cytokinesis (Marks et al., 1992; Fankhauser and Simanis, 1993). Under restrictive temperature conditions, the late cell plate mutants, *cdc3, cdc4, cdc8*, and *cdc12*, become elongated and dumbbell shaped, accumulate up to eight nuclei, and assemble ill-formed septa. Ultrastructural studies have suggested that the products of these genes might be required for normal F-actin function (Streiblová et al., 1984); a proposal supported by the characterization of one member of the late cell plate category, *cdc8*.

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(Balasubramanian et al., 1992). The cdc8* gene has been cloned and its product has been identified as a novel tropomyosin essential for F-actin contractile ring function (Balasubramanian et al., 1992).

In this paper, we describe the molecular cloning and characterization of another member of the late cell plate category, cdc3*. We find that the cdc3* gene encodes a profilin. Profilins were identified initially as actin-monomer sequestering proteins (Markey et al., 1981). Subsequent analyses, however, have shown that the functions of profilin are more complex. Profilins seem to perform a variety of functions including actin-monomer sequestration (Markey et al., 1981), actin filament formation (Pantaloni and Carlier, 1993; Haarer et al., 1990; Cooley et al., 1992) and growth factor-mediated signal transduction to the actin cytoskeleton (Machesky and Pollard, 1993). In S. pombe Cdc3-profilin is required for formation of an F-actin contractile ring. When overproduced, however, Cdc3-profilin causes the loss of all detectable F-actin structures. These experiments provide in vivo evidence for the duality of profilin function described earlier from biochemical studies of profilins.

Materials and Methods

Strains, Media, Cell Synchronization, and Genetic Methods

Schizosaccharomyces pombe strains used in this study and their origins have been summarized in Table I. Media for growth of yeast cells and general yeast genetic methods were as described by Moreno et al. (1991). (Represers of transcription from nmt promoter (Maundrell, 1993), was a generous gift of Drs. B. Edgar and C. Norbury. Plasmid REFX covers the budding yeast LEU2 gene, which complements S. pombe leu-22 mutants, as a selectable marker. This cDNA library was introduced into S. pombe cells, and selection was applied directly for leucine prototroph, as well as growth under restrictive temperature conditions. 32 colonies meeting these criteria were obtained. Subsequent analysis confirmed that all of these plasmids carried cDNAs representing the cdc3* locus.

Molecular Biology Methods

Standard techniques for DNA manipulation and bacterial transformations were used (Sambrook et al., 1989). The cdc3* gene was sequenced as follows. Subclones derived from the 1-kb SacI-BamHI fragment, which was previously determined to carry the cdc3-124 rescuing activity, were created in phagemid vectors pTZ18R and pTZ19R (Pharmacia, Uppsala, Sweden). Single-stranded DNAs corresponding to these subclones were generated, and nucleotide sequences were determined using the SequiPrep version 2.0 (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instruction. The SwissProt database was searched using the FASTDB program (Brutlag et al., 1990) with the predicted amino acid sequence of Cdc3p as the query sequence.

Molecular Cloning of the S. cerevisiae PFY1 Gene

Complementary DNA corresponding to the budding yeast gene encoding profilin was amplified by polymerase chain reaction from an S. cerevisiae cDNA library constructed in the plasmid pRS316 (Liu et al., 1992). This library was kindly provided to us by Dr. Anthony Weil. Primers were designed based on the published sequence of the budding yeast PFY1 gene (Magdolos et al., 1988). The cleavage site for the restriction enzyme BamHI was also included in each primer to facilitate cloning of the PFY1 gene as a BamHI fragment. The amplified product was cloned into pSK(+)(Strategene, La Jolla, CA), and its sequence was verified. Subsequently PFY1 was moved as a BamHI-BamHI fragment into the S. pombe expression vector pREP4 (Maundrell, 1993) to produce plasmid pMB310.

Deletion of the Cdc3 gene

Plasmid MB309 was created by deleting the Cia1-BamHI fragment carrying all but the NH-terminal four amino acids from the predicted cdc3* coding region (see Fig. 2 B) and replacing it with a 1.8-kb fragment carrying the S. pombe ura4* gene. pMB309 was linearized by digestion with KpnI and SpII, and a 4.7-kb fragment carrying the cdc3-ura4* DNA was purified after agarose gel electrophoresis. This linear fragment was used to transform the uracil auxotrophic diploid strain MY34, and selection was applied for colony formation on agar plates lacking uracil. Transformants were replica plated five times at 1-d intervals to media containing uracil to allow loss of any autonomously replicating DNA molecules carrying the ura4* gene. Cells were subsequently replica plated to medium lacking uracil, and clones that still remained prototrophic for uracil were treated as putative stable integrants. Genomic Southern blot analysis and analysis of spores confirmed that one of these strains was heterozygous at the cdc3* locus of the genotype cdc3-dispA:ura4*.

Expression in E. coli

Cdc3p was expressed in E. coli as two different fusion proteins. The full-length cdc3*-cDNA was amplified by polymerase chain reaction using oligonucleotides 5'-TGGCAAATGATCTTGG-3' and 5'-TTTGCATGCAAAATCT-3'. The amplified product was treated with Klenow enzyme to produce blunt ends and inserted into pTZ19R at the Smal site. Subsequently, cdc3*-cDNA was moved as a BamHI-EcoRI fragment into the E. coli expression vectors pGEX2T (Smith and Johnson, 1988) and pKSETA (Inviv...
trogen, San Diego, CA) to produce plasmids pKG251 and pKG233, which expressed Cdc3p as a fusion with glutathione S-transferase (GST) and a polyhistidine track (His6) containing peptide respectively. Pilot experiments showed that both fusion proteins, GST-Cdc3p and His6-Cdc3p, were synthesized at high levels and were soluble in aqueous solutions. The GST-Cdc3p fusion protein was purified over a glutathione agarose affinity column, and the His6-Cdc3p fusion protein was purified taking advantage of the high affinity of His6 to Ni2+.

**Antibodies**

Polyclonal antisera were raised in two different rabbits (FLOrence and CLEOpatra) as follows. In each case, 750 μg of soluble His6-Cdc3p fusion protein was emulsified with an equal volume of Freund’s incomplete adjuvant and injected subcutaneously into these rabbits. Booster injections with 100 μg of fusion protein emulsified in Freund’s complete adjuvant were administered at monthly intervals. Antibodies specific to Cdc3-profilin were purified from an antigen affinity column. GST-Cdc3p fusion protein was covalently coupled to cyanogen bromide-activated Sepharose beads and packed in a column. Serum from each rabbit was passed through this column to allow binding of antibodies specific to Cdc3-profilin to the affinity matrix. After washing the column extensively, antibodies bound to the affinity matrix were eluted in fractions into tubes containing 0.1 M Tris, pH 8.0, with 0.15 M glycine, pH 2.5. The purity of the eluted fractions was assessed by immunoblotting. Fractions containing Cdc3-profilin-specific antibodies were pooled. The antibody concentration was <5 μg/ml. To this pool, fetal calf serum was added to 0.1% wt/vol final concentration, and aliquots were frozen at −70°C. Immunoglobulins from the preimmune sera were purified on a protein A-Sepharose column as described in Harlow and Lane (1988). They were used for immunofluorescence at 1 μg/ml final concentration.

**Protein and Immunoblot Analyses**

Proteins were isolated from cells by disrupting cell walls with glass beads and extracting the released proteins with phosphate-buffered saline containing 1% NP-40, 2 mM EDTA, and protease inhibitors. Unless otherwise specified, proteins were separated on 6–20% polyacrylamide gradient gels containing SDS. After electrophoresis, the separated proteins were transferred by electroblotting to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). These blots were challenged with affinity-purified antiserum FLO at 1:400 dilution, incubated with 125I-labeled protein A, and specific reactions were detected by autoradiography.

**Fluorescence Microscopy**

Calcofluor/DAPI staining was accomplished as follows. Cells from a midlog cell wall material are visualized simultaneously although staining of nuclei was substantially masked by the bright fluorescence emanating from excitation of calcofluor. To facilitate clear visualization of nuclei and cell wall material, photographs were first taken immediately after mounting for calibration of calcofluor. To facilitate clear visualization of nuclei and cell wall material, photographs were first taken immediately after mounting for calibration of calcofluor-stained images and after passage of a few minutes for DAPI-stained images, since fluorescence emitted from calcofluor was found to fade rapidly. Staining of F-actin with rhodamine-conjugated phallolidin was performed as described (Marks and Hyams, 1985). For immunostaining, cells were fixed with a mixture of formaldehyde and glutaraldehyde (Moreno et al., 1991) and stained with affinity-purified anti-Cdc3 antibodies or preimmune immunoglobulins followed by a Texas red-conjugated goat anti-rabbit IgG secondary antibody. In all cases photographs were taken using a camera mounted on the microscope with Tri-X pan 400 ASA film (Eastman Kodak Co., Rochester, NY) and printed on Agfa Rapidine P1-4 paper.

1. Abbreviations used in this paper: DAPI, 4',6-diamidino-2-phenylindole; GST, glutathione-S-transferase; His6, polyhistidine track.
Figure 1. F-actin and septum material staining in cdc3-124 mutants. Cells were grown at 25°C to midlog growth phase and the culture was split in two. One half was shifted to 36°C, and the other half was allowed to continue growth at 25°C. After 4 h, cells were fixed and stained for F-actin with rhodamine-conjugated phalloidin or for septum material with calcofluor. In all cases, DAPI was included to visualize the nuclei. (A) DAPI staining of cells grown at 25°C; (B) F-actin staining in the cells shown in A; (C) DAPI staining in cells arrested at 36°C; (D) F-actin staining of cells in C; (E) DAPI staining in cells grown at 25°C; (F) septum staining of cells in E; (G) DAPI staining in cells arrested at 36°C; (H) septum staining of cells in G.
nucleotide sequences were determined. This analysis confirmed the predicted position of the two introns.

Comparison of the predicted 127-amino-acid polypeptide sequence with those in available databases suggested that the cdc3+ gene encodes a profilin (Fig. 3). The profilin encoded by the budding yeast PFYI gene (Magdolen et al., 1988) was most similar to the Cdc3-profilin. These proteins were 53% identical in sequence, and they were 76% similar to each other when conservative amino acid substitutions were considered. Cdc3-profilin was 48% identical to profilins from Acanthamoeba (Pollard and Rimm, 1991), 45% identical to Physarum profilins (Binnette, 1990), and 36% identical to Drosophila profilin (Cooley et al., 1992). Profilins from vertebrates are 140 amino acids in length and are less related to profilins from invertebrates and unicellular eukaryotes (Kwiatkowski and Bruns, 1988; Sri Widada et al., 1989). Two insertions are present when head-to-tail alignments are created between vertebrate profilin sequences and other profilin sequences.

**Construction and Analysis of a cdc3 Null Mutant**

To determine whether complete loss of cdc3 function would result in a cytokinesis defect similar to that of the temperature sensitive mutation, a null mutant of cdc3 was created. A DNA molecule was constructed in which the coding region of cdc3+ was replaced with the marker gene ura4+ (see Fig. 2 B and Materials and Methods). Linearized DNA containing this cdc3::ura4+ allele was used to transform a diploid (MBY34) to uracil prototrophy. Southern blot analysis confirmed that in one Ura+ transformant (MBY37), there had been a successful replacement of one of the wild-type copies by the cdc3::ura4 allele (Fig. 4 A). MBY37 was sporulated and spores were plated in rich media. Under these conditions ~50% of the spores generated colonies, all of which were Ura-. Ura+ colonies were obtained infrequently, all of which were subsequently confirmed to be unsporulated diploids that had survived the treatment by ascus wall/cell wall degrading enzymes. Thus, the cdc3+ gene is essential for cell proliferation.

Figure 2. Molecular cloning and nucleotide sequence of the cdc3 gene and the predicted amino acid sequence of Cdc3p. (A) Molecular cloning, restriction mapping, and complementation tests. Clones 3, 9, and 16 were isolated as plasmids that rescued the lethality of cdc3-124 cells. Restriction maps of the S. pombe genomic DNA in these plasmids were generated. Clone 16A1, which carries 1 kb of yeast genomic DNA retained its ability to rescue cdc3-124 mutant cells. The cdc3 coding region present in three exons is boxed. (B) Nucleotide sequence of the cdc3 gene and the predicted amino acid sequence of Cdc3p. Nucleotide position 1 denotes the start of the cdc3 coding region. The coding region is present in three exons. Intron sequences are shown in lower case letters. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z30648.
### Figure 3
Alignment between the predicted amino acid sequence of Cdc3p and other profilins. Amino acids identical in all profilins have been boxed. All profilins considered in this figure, with the exception of Cdc3p, are 125 or 126 amino acids in length. Therefore, gaps have been introduced in these protein sequences to maximize alignment. See text for references to the various profilin sequences.

| Cdc3p | S. pombe | S. cerevesiae | Acanthamoeba IA | Acanthamoeba II | Physarum P | Physarum A | Drosophila |
|-------|----------|---------------|-----------------|----------------|------------|------------|------------|
|       | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E | M S W Q A V Y T C T L G T K I D R A A V S R A G D S V W A A S A G F N L S P Q E |
|       |          |               |                 |                 |            |            |            |

### Figure 4
Analysis of a cdc3-null mutant. (A) Genomic Southern blot analysis to confirm deletion of a copy of the cdc3 gene. Genomic DNA was isolated from a cdc3*/cdc3- strain (SP34) and a stable Ura+ integrant potentially of the genotype cdc3::ura4/cdc3+ (SP37). The DNA was digested with BamHI, separated by electrophoresis, blotted, and probed with labeled cdc3 genomic sequences outside of the region deleted in cdc3::ura4. The 3-kb band represents the wild-type cdc3 locus and the 4.4-kb band represents the cdc3::ura4 locus. (B) F-actin staining and nuclear architecture in cdc3::ura4 haploids. cdc3::ura4 haploids rescued by virtue of a plasmid-borne cdc3 cDNA were grown under nonselective conditions to allow for loss of the plasmid, fixed, and stained with rhodamine-conjugated phalloidin and DAPI.

To test if cdc3-124 and cdc3::ura4 are mutant alleles of the same gene, a diploid strain of the genotype cdc3::ura4/cdc3- (MBY40) was constructed. MBY40 became temperature sensitive for colony formation at 36°C, whereas the otherwise isogenic strain, MBY37, whose genotype differed only at the cdc3 locus, was not. Analysis of spore products of MBY37 and MBY40 showed that in both cases, all viable colony forming spores were Ura-. Furthermore, all viable
Figure 5. Detection of Cdc3 profilin in wild-type cells, cells over-producing Cdc3 profilin, and in cdc3-124 mutants. (A) Immunoblot with anti-Cdc3p serum, FLO. Proteins were extracted from wild-type cells carrying no plasmid, carrying a multicopy plasmid containing the cdc3 gene (pUP profilin) or carrying a plasmid capable of producing highly elevated levels of Cdc3 profilin (pREP profilin). The extracted proteins were separated by electrophoresis, blotted on to nylon membranes, challenged with antigen-affinity purified antiserum (FLO), and autoradiograms were generated after treatment of blots with "125I-labeled protein A. (B) The cdc3-124 mutation does not affect steady-state level of Cdc3-124 profilin. Proteins were extracted from cdc3-124 cells grown at 25°C or arrested at 36°C for 4 h and treated for immunoblot analysis as described above.

spores resulting from sporulation of MBY40 were temperature sensitive for colony formation at 36°C, whereas viable spore products of MBY37 were not. These experiments confirmed that the cloned gene encoding profilin is the wild-type allele of cdc3 and not a high dosage suppressor.

Terminal morphology of the presumed cdc3::ura4 spores suggested that the cdc3+ gene is not required for spore germination, since these spores elongated and arrested uniformly as elongated and dumbbell-shaped single cells (data not shown). To further analyze the effect of cdc3 gene deletion, cdc35 was introduced into MBY37 on a multicopy plasmid, and the transformant was sporulated. Haploid cdc3::ura4 cells, rescued by virtue of plasmid-borne cdc3+ cDNA were isolated (MBY62). These cells were grown under nonselective nutritional conditions to allow loss of the autonomously replicating cdc3+ expressing plasmid. Microscopic examination revealed that a proportion of the cells were elongated and dumbbell shaped. Nuclear staining revealed that the elongated and dumbbell-shaped cells accumulated up to eight nuclei. Staining for F-actin with rhodamine-conjugated phalloidin displayed the presence of F-actin in dots and patches but not in the medial contractile ring (Fig. 4 C). This phenotype was similar to that seen in temperature-arrested cdc3-124 cells (Fig. 1 D). Thus, lack of cdc3+ function results in an inability of cells to complete cytokinesis without affecting the nuclear division cell cycle.

Detection of Cdc3-Profilin

Affinity-purified anti-Cdc3 sera recognized a single band with an apparent relative molecular mass of 13,000 on immunoblots of total proteins prepared from wild-type S. pombe cells (Fig. 5 A). The intensity of this band increased when the level Cdc3 profilin expression was increased either by the introduction of a multicopy plasmid bearing the cdc3+ gene or by placement of the cdc3+ gene downstream from the strongly transcribed nmtl promoter (Maundrell, 1989) (Fig. 5 A). These observations established that the protein recognized by these antisera is the product of the cdc3+ gene.

We then asked if the inability of cdc3-124 cells to undergo cytokinesis is related to the instability and disappearance of the mutant protein at the nonpermissive temperature. As illustrated in Fig. 5 B, the steady-state level of Cdc3-124 mutant profilin is no different at 36°C, suggesting that the defect in cdc3-124 cells is not caused by the loss of Cdc3-124 profilin, but rather by the loss of its function.

Cdc3-Profilin Colocalizes with F-actin

Affinity-purified anti-Cdc3 sera, when used for indirect immunofluorescence, gave a strong staining pattern that was not observed when cells were stained with purified immunoglobulins from the corresponding preimmune serum (Fig. 6 A). Therefore, we were able to use these antibodies to determine the intracellular distribution of Cdc3 profilin relative to F-actin by indirect immunofluorescence.

Three predominant patterns of profilin distribution were observed in wild-type cells (Fig. 6 B). In small cells, immunoreactive material was observed at one end of the cell (Fig. 6 B), presumed to be the growing end of the cell. After "new end take off" (NETO) (Mitchison and Nurse, 1985), a point in the S. pombe cell cycle when cells begin to grow from both poles, immunoreactive material was present at both ends of the cell. Finally, in binucleate cells, immunoreactive material was present in the medial region as a broad band. Punctate staining was observed occasionally in other regions of the cells. The nature of this staining is unknown. The locations where the vast majority of immunoreactive material was present coincided with the locations where F-actin was visualized (Fig. 6 B). However, whereas F-actin was concentrated in dots, patches, and rings, Cdc3 profilin was more diffuse in its localization.

Overexpression of Cdc3-Profilin

To study the effect of overproduction of Cdc3 profilin in wild-type cells, cdc3+ cDNA was placed under control of the thiamine-repressible nmtl promoter (Maundrell, 1989), and it was integrated into the chromosome of a wild-type strain to create strain MBY39. Under conditions of transcriptional repression, MBY39 cells were phenotypically no different than wild-type cells. Removal of thiamine resulted in a 10-15-fold overproduction of Cdc3 profilin (Fig. 5 A, pREP profilin). Microscopic examination revealed that the majority of Cdc3 profilin-overproducing cells arrested as elongated and dumbbell-shaped cells, with a phenotype reminiscent of that produced by cdc3-124 mutants at the restrictive temperature. When stained with anti-Cdc3p serum, these cells appeared intensely fluorescent, with most of the staining being present in the bulbous ends (Fig. 7 A and B). To study the correlation between localization of F-actin and Cdc3 profilin, MBY39 cells were stained with rhodamine-conjugated phalloidin. As a control, wild-type cells were stained simultaneously (Fig. 7 C). Interestingly, we ob-
Figure 6. Cdc3-profilin and F-actin colocalize. (A) Demonstration of specificity of affinity-purified FLO antiserum. Wild-type cells were immunostained with either purified immunoglobulins from preimmune serum at 1 μg/ml or with primary antiserum FLO at <1 μg/ml followed by Texas red-conjugated secondary antibodies. (B) Staining of Cdc3-profilin and F-actin in wild-type cells. Logarithmically growing wild-type cells were fixed and stained with rhodamine-conjugated phalloidin or with antiserum FLO followed by Texas red-conjugated secondary antibodies. In all cases, DAPI was also included to visualize the nuclei.
Figure 7. Phenotypic analyses of cells overproducing profilins. Cells overproducing Cdc3-profilin (MBY39) were fixed and stained with anti-Cdc3p antiserum (FLO), followed by Texas red–conjugated secondary antibodies. Cells overproducing profilins or overproducing both profilin and actin were fixed and stained with rhodamine-conjugated phalloidin to visualize F-actin architecture in these cells. (A) MBY39 cells stained with anti-Cdc3p serum (FLO). (B) DAPI staining of cells in A; (C) wild-type cells stained with rhodamine-conjugated phalloidin; (D) MBY39 cells stained with rhodamine-conjugated phalloidin; (E) MBY39 cells carrying pACT7-2 stained with rhodamine-conjugated phalloidin; (F) wild-type cells overproducing S. cerevisiae Pfyl-profili stained with rhodamine-conjugated phalloidin.
Actin monomers causing a deprivation of the pool of poly-
merization-competent actin monomers. If this was the rea-
served no specific fluorescent staining in cells overproducing
Cdc3-profilin in the rhodamine channel (Fig. 7 D). No dots,
patches, or rings were detected.

One hypothesis to explain the lack of observable F-actin
structures in MBY39 is that high levels of profilin sequester
actin in these cells. Upon replica plating to medium
lacking thiamine, we found that the cells carrying the control
plasmid became elongated and dumbbell shaped, and were similar in appear-
ance to those overexpressing the S. pombe Cdc3-profilin. Staining of cells carrying pMB310 with rhodamine-conju-
gated phalloidin failed to reveal detectable F-actin structures (Fig. 7 F).

Molecular Analyses of the Cdc3-124 Mutant Profilin

The cdc3-124 mutant gene was cloned after amplification of the
cdc3 locus from DNA prepared from a cdc3-124 strain.
Nucleotide sequence of the cdc3-124 gene identified a single
G→A change, consistent with the mutagenic potential of
nitrosoguanidine, which was used as a mutagen to isolate the
cdc3-124 mutant (Nurse et al., 1976). This G→A transition changed codon 43 in the cdc3 coding region from GAA to
AAA, resulting in a substitution of lysine for the glutamic acid residue found in this position (E43K).

To overproduce the Cdc3-124 mutant profilin, a cDNA
carrying the E43K mutation was generated and cloned downstream of the nmt1 promoter to create plasmid MB312.
Plasmid MB312 was introduced into wild-type (KGY246) and
cdc3-124 (KGY433) cells. Interestingly, we found that pMB312 was capable of allowing KGY433 cells
to form colonies at conditions restrictive for cdc3-124 mu-
tants. This rescue was observed only when transcription
from the nmt1 promoter was derepressed by removal of thia-
mie, suggesting that an excess of the E43K mutant profilin
was required for rescue of cdc3-124 mutants. At a low level
of penetrance, cell proliferation was affected in both wild-
type and cdc3-124 cells overproducing the Cdc3-124 mutant
profilin, since colonies were found to contain a proportion
of dead and lysed cells.

We stained KGY246 and KGY433 cells overproducing the mutant E43K mutant profilin with rhodamine-conju-
gated phalloidin. Surprisingly, F-actin was visualized in the form
of broad bands and big patches in both wild-type (KGY246) and
cdc3-124 mutant (KGY433) cells overproducing the E43K-mutant profilin at 36°C (restrictive temperature for
cdc3-124 mutants) (Fig. 8, A and B). Such staining has not
been observed in wild-type or any mutant cells studied thus
far. It is therefore possible that the low penetrance lethality

Analysis of Saccharomyces cerevisiae Pfyl-Profilin
Function in S. pombe

We isolated a cDNA encoding the budding yeast Pfyl-profilin for two reasons. First, we wanted to assess the ability of Pfyl-profilin, which is most closely related to in sequence to Cdc3-profilin, to rescue the lethality of a cdc3-124 mu-
tant. Second, we wanted to determine if overexpression of Pfyl-profilin in S. pombe cells produced a phenotype similar to that exhibited by cells overproducing Cdc3-profilin. To ex-
executing these experiments, the S. cerevisiae Pfyl cDNA was placed downstream of the nmt1 promoter in pREP4 to pro-
duce plasmid pMB310.

Plasmid MB310 was introduced into cdc3-124 ura4-D18 cells (KGY435), and colonies were allowed to form at 25°C in medium containing thiamine, to allow repression of trans-
scription of the Pfyl gene. These colonies were replica
plated to a range of thiamine concentrations for a 20-h period to allow for different levels of Pfyl-profilin expression, and
were then shifted to restrictive temperature conditions.
KGY435 cells carrying pMB310 were found to be incapable of colony formation at any level of expression. Thus, the S.
cerevisiae Pfyl-profilin appears to be incapable of providing
the essential function performed by the S. pombe Cdc3-
profilin.
in cells overproducing the mutant profilin is a consequence of the unusual F-actin structures found in these cells.

Discussion

This study has identified the product of the S. pombe cdc3* gene as a profilin, a known monomeric actin binding protein. Structural and functional features that are shared between Cdc3p and two other known profilins show that the product of the cdc3* gene is an authentic profilin. First, Cdc3p is 53, 48, and 36% identical in sequence to the budding yeast Pfyl-profilin (Magdolen et al., 1988), the Acanthamoeba profilins (Pollard and Rimm, 1991) and the Drosophila chickadee profilin (Cooley et al., 1992) respectively. Second, expression of cDNA encoding the Drosophila chickadee gene completely reverses the lethality associated with loss of Cdc3-profilin, suggesting that the Drosophila profilin can perform all the essential functions performed by Cdc3-profilin (M. K. Balasubramanian, K. Edwards, D. Kiehart, and K. L. Gould, unpublished observations).

Why is profilin essential for cytokinesis in S. pombe? Analysis of the F-actin cytoskeleton of cdc3-124 and cdc3-null mutants suggest that the defect in cytokinesis in these mutants results from the inability of these cells to synthesize and/or maintain a F-actin contractile ring. The contractile ring forms in the medial plane of the cell in a temporally and spatially regulated manner, being formed around midmitosis and disassembled after cytokinesis (Marks and Hyams, 1985). Although earlier biochemical studies postulated that profilin might act as an actin monomer sequestering protein (Markey et al., 1981), recent studies suggest that profilin can also function to promote actin filament assembly (Pantaloni and Carlier, 1993). It is known that ATP-bound actin monomers polymerize more rapidly than ADP-bound actin monomers, and profilin catalyzes the exchange of nucleotide on actin (Goldschmidt-Claremont, 1992). Recent studies have also shown that profilin can promote actin filament formation by lowering the critical concentration of actin (Pantaloni and Carlier, 1993). Thus, Cdc3-profilin might contribute to the formation of the F-actin contractile ring by either or both of these mechanisms. The possibility that Cdc3-profilin participates in F-actin contractile ring formation is supported by our immunolocalization experiments that showed the presence of Cdc3-profilin in the medial region of the cell where the F-actin ring will form during cytokinesis.

At least two previous studies have also provided evidence for a requirement for profilin in actin filament formation in vivo. First, budding yeast cells deleted for the profilin encoding gene PFYI lack F-actin cables and undergo random budding and cytokinesis (Haarer et al., 1990). Second, Drosophila mutants that are deficient for an oocyte specific profilin transcript are defective for cytoplasmic actin networks leading to defective intracellular cytoplasm transport and sterility (Cooley et al., 1992).

Analysis of the terminal phenotypes of cdc3-124 and cdc3-null mutants illustrated that these mutants are impaired primarily for progression through cytokinesis. Spore germination, DNA replication, and mitosis were all unaffected. This presents a paradox, since it is well known that actin filament formation is required for a wide range of cellular processes.

Two possible rationales can be used to reconcile to these observations.

One possible explanation is that many components of the cytoskeleton, even in lower eukaryotes, are present as multiple isoforms that appear to perform specialized cellular functions. Although only one profilin-encoding gene has been identified in both the yeasts, multiple profilin isoforms with different biochemical properties have been found in Acanthamoeba and Physarum (Pollard and Rimm, 1991; Binette et al., 1990). Thus, two profilin isoforms with different functions might be present in S. pombe cells, and the second hypothetical profilin might not be able to substitute for Cdc3-profilin in catalyzing the formation of the F-actin contractile ring.

The second explanation is that the requirement for profilin is rate limiting only for cytokinesis, and that this is the only stage in the cell cycle where the effects of its absence are apparent. In other words, the F-actin ring that forms within a short window of cell cycle time cannot form rapidly in the absence of Cdc3-profilin. However, F-actin required in other processes (Novick and Botstein, 1985) might be assembled more slowly and/or might be formed by mechanisms not involving Cdc3-profilin.

Profilin overexpression experiments presented in this paper provide strong in vivo evidence that profilin can act as an actin monomer sequestering protein. Cells overproducing Cdc3-profilin become elongated and dumbbell shaped, and the majority of these cells arrest at cytokinesis. Staining with rhodamine-conjugated phalloidin failed to detect any F-actin containing structures; the dots, patches, and the contractile ring found in wild-type cells were absent. The simplest interpretation of this observation is that profilin, when present above a certain level, loses some aspect of its regulation and acts as an actin monomer sequestering protein. The experiments involving overproduction of both actin and Cdc3-profilin support that actin monomer sequestration is the probable cause of the phenotype observed in cells overproducing Cdc3-profilin; 60-70% of these cells are morphologically wild type. Staining with rhodamine-conjugated phalloidin revealed that F-actin was once again present in dots, patches, and rings in these cells. A similar but complementary set of experiments performed in the budding yeast had earlier pointed to the ability of profilins to sequester actin monomers in vivo (Magdolen et al., 1993). Overproduction of the prototypic actin Actlp in budding yeast cells results in lethality (Shortle et al., 1982). This lethality can be reversed by the overproduction of Pfylp in cells overproducing Actlp (Magdolen et al., 1993). A role for profilin in sequestering actin monomers had also been proposed from studies in cultured human cells, where microinjection of profilin was shown to cause loss of F-actin structures (Cao et al., 1992).

We have identified the mutation in the Cdc3-124 mutant protein, since it represents the only available conditional mutation in any profilin studied thus far. This mutation results in substitution of a glutamic acid at position 43 by a lysine. The crystal structure of the Acanthamoeba profilin I isoform has been solved recently (Vinson et al., 1993). It has been shown that this protein, which is 48% identical in sequence to Cdc3-profilin, is composed of seven β sheets and three α helices (Vinson et al., 1993). Assuming a similar structure for Cdc3-profilin, the mutation identified in the Cdc3-124-
profilin maps to the second α helix. At present, the function of this region of profilin is unclear. Amino acid position 43 does not appear to be near the actin or phosphoinositide binding regions of profilin. One possibility is that this mutation alters the folding of the protein, rendering it inactive. This explanation seems reasonable since the phenotype of cells lacking Cdc3-profilin is very similar to the phenotype of cdc3-I24 cells under restrictive conditions. This explanation is also strengthened by the finding that overproduction of the E43K mutant profilin restores the threshold level of profilin activity required for cells to carry out cytokinesis, although these cells showed abnormalities at the level of profilin structure. At present, we cannot explain the phenotype of cells overproducing the E43K mutant profilin. Biochemical characterization of the wild-type and E43K mutant will be necessary to understand the defect in the E43K mutant profilin.

In conclusion, this study has provided in vivo evidence for two diverse biochemical functions performed by profilins, actin filament formation and actin monomer sequestration. In addition, this study has also identified an amino acid residue essential for profilin function in vivo. With the availability of a conditionally lethal mutant in Cdc3-profilin, it should be possible to identify genes whose products might interact with Cdc3-profilin. We have initiated genetic suppressor analyses and indeed have isolated several extragenic suppressors of the cdc3-I24 mutation. Further analyses of these suppressors is in progress. Analysis of profilin structure/function relationships should also be facilitated, since the inability to perform cytokinesis can be used as a criterion to isolate additional mutations in this gene. These studies, together with the wealth of biochemical studies and genetic studies in budding yeast and flies, should help us to understand more precisely the roles of profilins.

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References

Balasubramanian, M. K., D. M. Helfman, and S. M. Hemmingsen. 1992. A new tropomyosin essential for cytokinesis in the fission yeast S. pombe. Nature (Lond.) 360:84-87.

Barbet, N., W. J. Muriel, and A. M. Carr. 1992. Versatile shuttle vectors and genomic libraries for use with Schizosaccharomyces pombe. Gene. 114:59-66.

Binnerte, F., M. Bénard, A. Larroche, G. Pierron, G. Lemieux, and D. Pallotta. 1990. Cell-specific expression of a profilin gene family. DNA Cell Biol. 9:333-334.

Brutlag, D. L., J. P. Daucourt, S. Maulik, and J. Relph. 1990. Improved sensitivity of biological sequence database searches. Comput. Appl. Biosci. 6:237-245.

Cao, G., G. G. Babcock, P. A. Rubenstein, and Y.-L. Wang. 1992. Effects of profilin and profiliaXin on actin structure and function in living cells. J. Cell Biol. 117:1023-1029.

Cooley, E., L. Verheyen, and K. Ayers. 1992. chickadee encodes a profilin required for intercellular cytoplasm transport during Drosophila oogenesis. Cell. 69:173-184.

Creanor, J., and J. M. Mitchison. 1979. Reduction of perturbations in leucine incorporation in synchronous cultures of Schizosaccharomyces pombe. J. Gen. Microbiol. 112:385-391.

Fankhauser, C., and V. Simanis. 1993. The Schizosaccharomyces pombe cdc4 gene is required for septum formation and can also inhibit nuclear division. Mol. Biol. Cell. 4:531-539.

Goldschmidt-Clermont, P. J., M. I. Furman, D. Wachsstock, D. Safer, V. T. Nachmanis, and T. D. Pollard. 1992. The control of actin nucleotide exchange by thymosin B4 and profilin. A potential regulatory mechanism for actin polymerization in cells. Mol. Biol. Cell. 3:1015-1024.

Harley, C., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 312-315.

Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdolen, W. Bandlow, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110:105-114.

Hoheisel, J. D., E. Maier, R. Mott, L. McCarthy, A. V. Grigoriev, L. C. Schallw, D. Nizetic, F. Francis, and H. Lehrach. 1993. High-resolution cosmid and PI maps spanning the 14Mbp genome of the fission yeast Schizosaccharomyces pombe. Cell. 73:109-120.

Jochová, J., K. Rupes, and E. Streiblová. 1991. F-actin contractile rings in protoplasts of the yeast Schizosaccharomyces. Cell Biol. Int. Rep. 15:607-610.

Kwiatkowski, D. J., and G. A. P. Bruns. 1988. Human profilin: molecular cloning, sequence analysis, and chromosomal analysis. J. Biol. Chem. 263:5910-5915.

Lennon, G. G., and H. Lehrach. 1992. Gene database for the fission yeast Schizosaccharomyces pombe. Curr. Genet. 1:1-11.

Liu, H., J. Krizek, and A. Bretscher. 1992. Construction of a GAL1-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics. 132:665-673.

MacKoshy, L. M., and T. D. Pollard. 1993. Profilin as a potential mediator of membrane-cytoskeleton communication. Trends Cell Biol. 3:381-385.

Maier, E. J., D. Hoheisel, L. McCarthy, R. Mott, A. V. Grigoriev, A. P. Monaco, Z. Larin, and H. Lehrach. 1992. Complete coverage of the Schizosaccharomyces pombe genome in yeast artificial chromosomes. Nature Genet. 1:273-277.

Magdolen, V., U. Oechsner, G. Müller, and W. Bandlow. 1988. The intron-containing gene for yeast profilin (PFF) encodes a viral factor. Mol. Cell. Biol. 8:5108-5115.

Magdolen, V., D. G. Drubin, G. Mages, and W. Bandlow. 1993. High levels of profilin suppress the lethality caused by overproduction of actin in yeast cells. FEBS (Fed. Eur. Biochem. Soc.) Lett. 316:41-47.

Marks, T. F., T. Persson, and B. Edgard. 1981. Characterization of plamid extracts before and after stimulation with respect to the possible role of profilin activity as microfilament precursor. Cell. 23:145-153.

Marks, J., C. Fankhauser, and V. Simanis. 1992. Genetic interactions in the control of septation in Schizosaccharomyces pombe. J. Cell Sci. 101:801-808.

Marks, J., J. M. Hagen, and J. S. Hyams. 1987. Spatial association of F-actin with growth polarity and septation in the fission yeast Schizosaccharomyces pombe. Spec. Publ. Soc. Gen. Microbiol. 23:119-135.

Marks, J., and J. S. Hyams. 1985. Localization of F-actin through the cell division cycle of Schizosaccharomyces pombe. Eur. J. Cell Biol. 39:27-32.

Maundrell, K. 1989. nm1 of fission yeast: a highly expressed gene completely suppressed by thymine. J. Biol. Chem. 265:10857-10864.

Maundrell, K. 1993. Thiamine-repressible vectors pREP and pRIP for fission yeast. Gene. 123:127-130.

Mitchison, J. M., and P. Nurse. 1985. Growth in cell length in the fission yeast Schizosaccharomyces pombe. Spec. Publ. Soc. Gen. Microbiol. 23:119-135.

Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. Cell. 40:405-416.

Nurse, P. 1985. Cell cycle control genes in yeast. Trends Genet. 1:51-55.

Nurse, P., P. Thuriaux, and K. Nasmyth. 1976. Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. Mol. Gen. Genet. 146:167-178.

Pantaloni, D., and M.-F. Carlier. 1993. How profilin promotes actin filament assembly in the presence of thymosin B4. Cell. 75:1007-1014.

Polland, T. D., and D. L. Rimm. 1991. Analysis of cDNA clones for Acanthamoeba profilin-I and profilin-II shows end to end homology with vertebrate profilins and a small family of profilin genes. Cell Motil. Cytoskel. 20:169-177.
Prentice, H. L. 1991. High efficiency transformation of Schizosaccharomyces pombe by electroporation. *Nucleic Acids Res.* 20:621.

Russell, P. 1989. Gene cloning and expression in fission Yeast. In Molecular Biology of the Fission Yeast. A. Nasim, P. Young, and B. F. Johnson, editors. Academic Press, Inc., San Diego, CA, pp. 244–271.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shortle, D., J. E. Haber, and D. Botstein. 1982. Lethal disruption of the yeast actin gene by integrative DNA transformation. *Science (Wash. DC)*. 217:371–373.

Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene.* 67:31–40.

Sri Widada, J., C. Ferraz, and J.-P. Liautard. 1989. Total coding sequence of profilin cDNA from *Mus musculus* macrophage. *Nucleic Acids Res.* 17:2855.

Streiblová, E., J. Hasek, and E. Jelke. 1984. Septum pattern in ts mutants of *Schizosaccharomyces pombe* defective in genes cdc3, cdc4, cdc8, and cdc12. *J. Cell Sci.* 69:47–65.

Vinson, V. K., S. J. Archer, E. E. Lattman, T. D. Pollard, and D. A. Trochia. 1993. Three-dimensional solution structure of *Acanthamoeba* profilin I. *J. Cell Biol.* 122:1277–1283.

Balasubramanian et al. *A Profilin Essential for Cytokinesis in S. Pombe*