cdc12p, a Protein Required for Cytokinesis in Fission Yeast, Is a Component of the Cell Division Ring and Interacts with Profilin

Fred Chang,*‡ David Drubin,‡ and Paul Nurse*

*Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom; and ‡Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3202

Abstract. As in many other eukaryotic cells, cell division in fission yeast depends on the assembly of an actin ring that circumscribes the middle of the cell. *Schizosaccharomyces pombe cdc12* is an essential gene necessary for actin ring assembly and septum formation. Here we show that cdc12p is a member of a family of proteins including *Drosophila* diaphanous, *Saccharomyces cerevisiae* BNI1, and *S. pombe* fus1, which are involved in cytokinesis or other actin-mediated processes. Using indirect immunofluorescence, we show that cdc12p is located in the cell division ring and not in other actin structures. When overexpressed, cdc12p is located at a medial spot in interphase that anticipates the future ring site. cdc12p localization is altered in actin ring mutants. *cdc8* (tropomyosin homologue), *cdc3* (profilin homologue), and *cdc15* mutants exhibit no specific cdc12p staining during mitosis. *cdc4* mutant cells exhibit a medial cortical cdc12p spot in place of a ring. *mid1* mutant cells generally exhibit a cdc12p spot with a single cdc12p strand extending in a random direction. Based on these patterns, we present a model in which ring assembly originates from a single point on the cortex and in which a molecular pathway for the functions of cytokinesis proteins is suggested. Finally, we found that *cdc12* and *cdc3* mutants show a synthetic-lethal genetic interaction, and a proline-rich domain of cdc12p binds directly to profilin cdc3p in vitro, suggesting that one function of cdc12p in ring assembly is to bind profilin.

Cell division in many eukaryotic cells is accomplished through the action of an actin-based contractile ring (for reviews see Conrad and Schroeder, 1990; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). The positioning of this ring, which dictates the subsequent division plane, can determine cell size, shape, and orientation. Placement of the division plane is also critical during development, for instance, in the segregation of localized determinants (Rhyu and Knoblich, 1995; Horvitz and Herskowitz, 1992). Events in cell division must be coordinated with events in nuclear division to ensure that each daughter cell receives one intact genome. In animal cells, the coordination of cellular and nuclear division events appears to involve a mechanism in which the cleavage plane is determined by the mitotic asters (see Rappaport, 1986). It has been proposed that the mitotic asters send signals to the cell surface that induce contractile ring formation (Rappaport, 1986). Although a number of components of the contractile ring have been identified, little is known about the molecular mechanism of ring assembly at the plasma membrane and even less is known about the putative signals responsible for establishing the position of the ring.

The fission yeast *Schizosaccharomyces pombe* is an excellent organism for studying eukaryotic cell division at the molecular-genetic level (Fankhauser and Simanis, 1994; Chang and Nurse, 1996). Cell division in fission yeast requires an actin-based ring, which forms in early mitosis and shrinks in late mitosis as the septum cell wall material grows centripetally from the cell surface (Marks and Hyams, 1985). This ring may play two roles in cell division. First, it may mark the site of cell wall septum formation, and, second, it may play a contractile role in actively closing the plasma membrane. Although the ring clearly closes (Jochova et al., 1991; McCollum et al., 1995; this work), definitive evidence that the actin ring is contractile—that it exerts an active, contractile force—is still lacking. Alternatively, the actin ring may stabilize or guide the plasma membrane during septation. Conservation of ring components indicate that the *S. pombe* actin ring may be similar at a molecular level to the contractile ring in larger eukaryotes.

In fission yeast, it has been proposed that the actin ring and the subsequent division site are positioned in the middle of the cell by a signal from the premitotic nucleus

Please address all correspondence to Fred Chang, Department of Microbiology, Columbia University, 701 West 168th Street, New York 10032. Fax: (212) 305-1468. e-mail: fc99@columbia.edu

(Chang and Nurse, 1996). The positions of the nucleus and subsequent division site always coincide, even in mutant cells with a displaced nucleus, multiple nuclei, or altered cell polarity (Toda, T., personal communication; Marks et al., 1987; Snell and Nurse, 1994; Verde et al., 1995). In contrast with animal cells (Rappaport, 1986), in fission yeast, ring assembly and placement are independent of the mitotic spindle (Chang et al., 1996). Indeed, assembly of a medial ring can be induced in certain circumstances in interphase cells (Fankhauser et al., 1995; Ohkura et al., 1995), suggesting that signals responsible for medial positioning may be present even before mitosis.

To study the molecular basis of actin ring assembly and placement, genetic screens have been used to identify six genes required for actin ring formation (*cdc3*, *cdc4*, *cdc8*, *cdc12*, *cdc15*, and *rng2*) and one gene required for ring placement (*mid1*) (Nurse et al., 1976; Marks et al., 1987; Chang et al., 1996). Temperature-sensitive mutations in these genes cause defects in cell division and actin organization at restrictive temperature. These mutants develop into large, multinucleate cells and ultimately die. Different aberrant patterns of actin distribution indicate that these gene products play different roles in actin ring assembly. *cdc3* and *cdc8* mutants display disorganized actin structures throughout the cell cycle, suggesting that *cdc3* and *cdc8*, which encode profilin and tropomyosin, respectively, may organize actin in a general fashion (Marks et al., 1987; Balasubramanian et al., 1992, 1994; Chang et al., 1996). *cdc12* and *cdc15* mutants exhibit delocalized actin patches during mitosis but normal actin distributions during interphase (Fankhauser et al., 1995; Chang et al., 1996), suggesting that these gene products play specific roles in ring assembly during mitosis. *cdc4* and *rng2* mutants display disorganized actin cables instead of a ring, suggestive of a defect in a late step of ring assembly (McCollum et al., 1995; Chang et al., 1996). *cdc4* encodes an EF hand–containing protein with properties of a myosin light chain (McCollum et al., 1995). *mid1* is necessary for placement of the ring, since *mid1* mutants exhibit rings and septa located at random positions and angles on the cell surface (Chang et al., 1996). cdc15p and the polo kinase homologue plo1p have been implicated as important regulators of ring assembly in the cell cycle, since overexpression of either one is sufficient to generate a ring at any stage of the cell cycle (Fankhauser et al., 1995; Ohkura et al., 1995).

Here we describe the cloning and molecular characterization of one of the actin ring genes, *cdc12.* The phenotype of *cdc12*^{ts} mutants suggests that *cdc12* gene product is required specifically for actin ring assembly during mitosis and thus may be a target for cell cycle and spatial regulators of ring assembly (Chang et al., 1996). cdc12p is a member of a family of proteins that have been implicated in cytokinesis, suggesting that the function of cdc12p is conserved. cdc12p is the first member of this protein family to be localized to a cell division ring. Studies of cdc12p localization patterns upon overexpression and in mutant backgrounds reveal a medial cdc12p spot. This localization pattern may provide novel insights into mechanisms of ring formation and placement. Furthermore, we provide genetic and biochemical evidence suggesting that one function of cdc12p is to associate with another gene product required for ring formation, profilin cdc3p. Profilins are small actin-binding proteins that have been shown to bind monomeric actin, an actin-related protein complex, phospholipid PIP2, and proline-rich peptides such as poly-L-proline and VASP, and may regulate actin dynamics in the formation of F-actin structures (see Machesky and Pollard, 1993; Pollard, 1995; Machesky et al., 1994). These studies begin to address how protein–protein interactions build the actin ring.

Table I. Schizosaccharomyces pombe Strains Used in This Study

Name	Genotype	Reference
972	wild type h$^-$	Leopold, 1970
FC43	*ura4-D18 h*$^-$	P. Nurse collection
FC20	*cdc12-112 ura4 h*$^-$	This work
FC127	*cdc12-229 h*$^-$	Chang et al., 1996
FC131	*cdc8-346 h*$^-$	Chang et al., 1996
FC55	*cdc15-287 ura4-D18 h*$^+$	Chang et al., 1996
FC361	*cdc4-377 h*$^-$*/h*$^-$ (diploid)	Chang et al., 1996
FC164	*mid1-366 h*$^-$	Chang et al., 1996
FC291	*ura4-D18 h*$^-$ pnmt-cdc12 (ura4$^+$)	This work
FC565	*ura4-D18 h*$^-$ pnmt-cdc12 integrated	This work
FC315	*ura4-D18 h*$^-$ pUR19 (ura4$^+$)	This work
FC335	*cdc12*$^+$*/cdc12::ura4 ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210 h*$^+$*/h*$^-$	This work
PN1419	*cdc25-22 leu1-32 h*$^-$	P. Nurse collection

Materials and Methods

Yeast Strains and Genetic Methods

Standard *S. pombe* genetic techniques and media were performed as previously described in Moreno et al. (1991). All *S. pombe* strains were isogenic to 972 (Leupold, 1970). *S. pombe* strains used are listed in Table I. FC20 *ura4-D18 cdc12-112 h*$^-$ was isolated in a cross between *cdc12-112 h*$^-$ and *h*$^+$ *ura4-D18* (P. Nurse lab collection). *cdc4-377* homozygous diploid strain was isolated spontaneously from FC123; this diploid was used because of the higher percentage of well-defined actin aster structures in the diploid, but the same structures were present in a haploid strain as well. Yeast transformations were performed by the lithium acetate method (Moreno et al., 1991) or by electroporation (Kelly et al., 1993).

DNA Manipulations and Hybridizations

Standard DNA ligation and cloning procedures were used (Maniatis et al., 1982) with GeneClean from Bio101 (Vista, CA) and DNA prepared from Qiagen plasmid kits (Chatsworth, CA). Genomic yeast DNA was prepared as described (Moreno et al., 1991). Enhanced chemiluminescence (ECL)[1] Southern hybridization was carried out as recommended (ECL southern blotting; Amersham Corp., Arlington Heights, IL). In colony hybridizations, ^{32}P-labeled DNA probes were prepared using a Prime-It kit (Stratagene, La Jolla, CA).

cdc12 Cloning

The *cdc12* gene was cloned by complementation of the temperature-sensitive lethal phenotype of *cdc12-112.* A pUR19 (*ura4*$^+$)-based *S. pombe* genomic library (Barbet et al., 1992) was transformed into *ura4D18 cdc12-112* (FC112), and 5×10^4 ura$^+$ transformant colonies were screened by replica printing onto YE5S with phyloxin for growth at 36°C. Four colonies showed plasmid-dependent rescue. Isolation of plasmid DNA from two of these transformants identified two related plasmids, pFC103 and

1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; GST, glutathione-*S*-transferase; PLP, poly-L-proline.

pFC104. pFC103 complemented a *cdc12-112* strain upon retransformation and contained a 6-kb insert in pUR19. Deletion analysis showed that a central region was critical for activity (see Fig. 1 *A*). Three genetic criteria demonstrated that complementing activity was the *cdc12* gene: first, hybridization to an ordered cosmid blot (Hoheisel et al., 1993) showed that pFC103 mapped to left tip of chromosome I, at the corresponding location to which *cdc12* has been previously mapped genetically. Second, pFC110, which contains a 3-kb EcoRI *cdc12* insert from pFC103 and a *sup3-5* marker (from pSTA12), was integrated into the genome by homologous recombination. Random spore analysis showed that the plasmid integrated at the *cdc12* locus. Third, introduction of the COOH-terminal half of *cdc12* gene into *cdc12-112* does not confer rescue at 36°C in the majority of the cells, but confers rare stable gene conversion of the *cdc12-112* allele, suggesting that the genetic defect of *cdc12-112* resides in the COOH-terminal half of the protein.

cDNA and additional genomic DNA clones of *cdc12* were isolated to delineate the ends of the transcript and to test the existence of possible introns. pFC125, which contains a partial 2.5-kb *cdc12* cDNA that includes the 3′ half of the gene from the central EcoRI site, was identified by hybridization (Maniatis et al., 1982) from a *S. pombe* cDNA λYES library (generous gift from S. Elledge, Baylor University, Houston, TX; Elledge et al., 1991) using central 1.0- and 0.9-kb HindIII fragments as a probe. The insert in pFC125 was subcloned in TZ18R vector to produce pFC126 and pFC127, which were used in sequencing and further subcloning. pFC128, which contains a 2.4-kb *cdc12* cDNA covering the 5′ end of *cdc12*, was obtained by a PCR approach 5′ RACE (Gibco BRL, Gaithersburg, MD). 109ext1 (GCATCATTAGGAATATCA) was used as an initial gene-specific oligonucleotide along with a 5′ anchor oligonucleotide. Two cycles of PCR with internal primers 109ext2 (GGAAGGATCCGTTGACACAG-TTGAGGG) and then 109ext3 (CAATTCCATCGTTTGACC) allowed the amplification of a 2.4-kb fragment that was cloned into a TA KS cloning vector (gift from J. Wuarin, Imperial Cancer Research Fund, London, UK). pFC163, which contains a 5-kb genomic *cdc12* fragment covering the 5′ end and putative promoter region of the gene in pDB248, was cloned by hybridization from the Tamagawa and Noguchi *S. pombe* genomic DNA library (gift from T. Toda, Imperial Cancer Research Fund; Hirano et al., 1988).

Deoxynucleotide Sequencing

Deoxynucleotide sequencing was performed using an ALF automated sequence machine using a T4 polymerase sequencing kit from Pharmacia Biotech Inc. (Piscataway, NJ). Both *cdc12* cDNA clones pFC127 and pFC128 and a 5.5-kb *cdc12* genomic clone from XhoI to BamHI derived from pFC103 were sequenced, using subclones and primer walking. The pFC127 cDNA (3′ of EcoRI) and the pFC155 genomic DNA (5′ *cdc12* BamHI to EcoRI) were sequenced completely on both strands, and sequences from these were used when the cDNA and the genomic sequences did not agree; in particular, numerous single base pair changes were found in the pFC128 cDNA isolated by PCR, which were not incorporated. In addition, pFC163 was used to confirm sequences 5′ of the start site not included in pFC103 to delineate the start AUG. Sequences were aligned and analyzed using the DNA analysis programs Assemblign, DNA Strider, and DNA-Star.

cdc12::ura4 Deletion

A 5.5-kb BamHI–XhoI *cdc12* DNA fragment from pFC103 containing the entire *cdc12* open reading frame was ligated into the BamHI and XhoI sites of KS+ vector to produce pFC161. pFC162 (*cdc12::ura4*) was constructed by replacement of a 4.1 kb of PstI–SalI fragments in *cdc12* in FC161 by a 1.8-kb PstI–SalI *ura4* fragment. To replace the wild-type *cdc12* gene with *cdc12::ura4*, pFC162 was cut with BamHI and XhoI, and the upper 3.8-kb band was gel purified and transformed into an *ura4*/*ura4 leu1*/*leu1 ade6-M210*/*ade6-M216 h+*/*h−* diploid. Multiple *cdc12::ura4* heterozygous diploids were confirmed by Southern blotting and PCR. In random spore analysis, *cdc12::ura4* heterozygous diploids were grown in minimal media with leucine to late exponential phase, washed into minimal media with no nitrogen, allowed to sporulate at 25°C for 2 d, and digested with helicase (Moreno et al., 1991). The resultant spores were germinated in minimal media with adenine and leucine for 15 h at 30°C.

cdc12 Overexpression

pnmt-cdc12 was constructed by insertion of the moderate strength *nmt1* promoter from pREP41X into the KpnI–BamHI site 60 bp upstream of the *cdc12* open reading frame in pFC103 (*ura4+*). The *nmt1* promoter fragment was generated by PCR using a 5′ nmt promoter oligonucleotide OKS3 GGGGGTACCCGCCATAAAAGACAGAATAAG and a 3′ nmt oligonucleotide, cut with KpnI and BamHI. Both stable pnmt-cdc12 transformants (FC565) that had integrated the plasmid in the chromosome and unstable transformants (FC291) that contain free plasmid were analyzed and produced similar cdc12p localization patterns.

Generation of Anti-cdc12p Antibody

A His_6-tagged protein fragment of cdc12p purified from *Escherichia coli* was used for the production of the polyclonal antibodies. This protein was obtained in the following manner: a 0.9-kb *cdc12* fragment that encodes cdc12 FH1 domain was PCR amplified with the oligonucleotides 107rev (GGAAGGATCCAACACACTCGTTCATGC) and 5′ BamPst (GGA-AGGATCCGCTGCAGAGTCAACACTT), cut with BamHI, and inserted into the BamHI site of the His-tag expression vector pQE9 to make pFC122. pFC122 (multiple isolates analyzed) was transformed into *E. coli* M15/REP4. His_6-tagged protein from *E. coli* was purified on Ni agarose using a denaturing protocol from the insoluble fraction as recommended in the QIAexpress manual (Qiagen). 2 mg of purified protein in acrylamide gel slices or eluted in PBS was injected into two rabbits. One rabbit (FC2) produced sera that recognized a 220-kD protein in crude yeast extracts on an immunoblot. For affinity purification, FC2 sera were loaded over a Sepharose 4B column containing the purified denatured His_6-tagged cdc12p FH1 protein fragment and eluted with 4.5 M $MgCl_2$ and then 100 mM glycine (acid) (Pfeffer et al., 1983). Eluates were immediately dialyzed into PBS + 30% glycerol. $MgCl_2$ eluate was used for Western analysis, and the glycine eluate was used for immunofluorescence (described below).

Western Analysis

Crude boiled whole yeast cell extracts were prepared as described in Correa-Bordes and Nurse (1995). Protein concentrations were measured standardized both by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) and by Coomassie staining. 5–50 μg extract per lane was loaded on 6% SDS-PAGE and blotted to 0.2-μm nitrocellulose using Transfer Buffer I (Harlow and Lane, 1988) at 8 V for 16 h at 4°C. Blots were washed with H_2O, stained with Ponceau S, and processed as recommended using ECL Western blotting kit (Amersham Corp.) with anti-cdc12 affinity-purified antibodies at 1:500 dilution and TBS + 0.1% Tween.

Immunofluorescence

For immunofluorescence, cells were fixed in cold methanol and processed as described (Snell and Nurse, 1994). Anti-cdc12 antibody (1:10 dilution) and sheep anti–rabbit IgG CY3 conjugate secondary antibody (1:200 dilution; Sigma Chemical Co., St. Louis, MO) were used to visualize cdc12p. The anti-tubulin mAb TAT1 (1:5; generous gift from K. Gull, University of Manchester, Manchester, UK; Woods et al., 1989) or anti-actin mAb (1:200; Amersham Corp.) and anti–mouse FITC conjugate secondary antibody (1:200) were used to visualize microtubules and actin, respectively. Stained cells were mounted with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, Inc., Burlingame, CA). Rhodamine-phalloidin staining on formaldehyde-fixed cells was performed exactly as described previously (Chang et al., 1996). Cells were photographed as described in Chang et al. (1996) or with a Northern Exposure imaging system (Phase 3 Imaging Systems, Glen Mills, PA).

In Vitro Binding Experiments

pKG251 (Balasubramanian et al., 1993) was a gift from K. Gould and M. Balasubramanian (University of Tennessee, Nashville). Soluble glutathione-*S*-transferase (GST)–cdc3p and GST proteins were expressed from pKG251 and pGAT2 in *E. coli* strain BL21 and purified on glutathione beads. His_6-tagged cdc12p was expressed from pFC122 (described above), purified under denaturing conditions, and renatured by dialysis from 8 M urea-PBS in stepwise fashion into PBS. cdc12p protein solution was precleared before binding assays by a centrifugation at 14K rpm for 10 min in a microfuge. Final concentration of cdc12p protein was 0.6 mg/ml. Approximate molecular masses of the proteins were: GST-cdc3 = 40 kD and cdc12p = 45 kD. Poly-L-proline (PLP; 1,000–10,000; average mol wt 5,600; n = 50; Sigma Chemical Co.) was diluted in PBS. In binding experiments, 20 μl of appropriate dilution of cdc12p in PBS, 20 μl of 50% beads in PBS

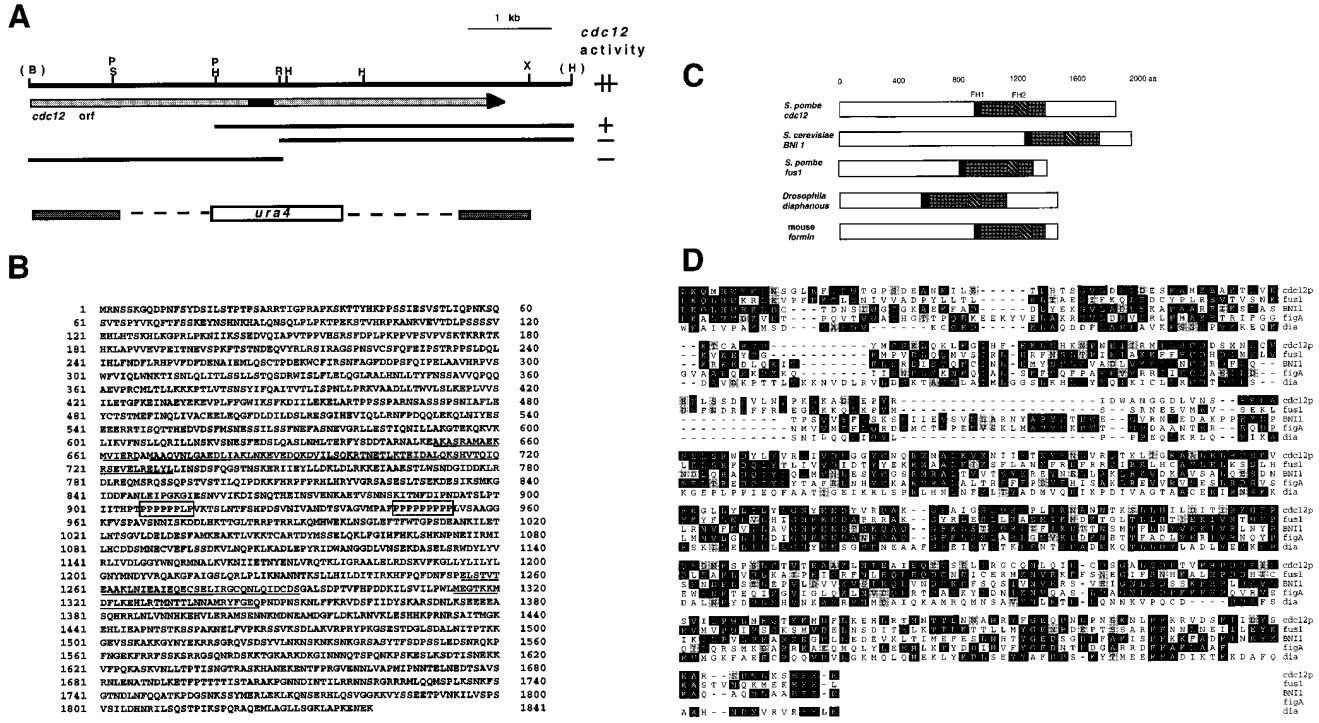

Figure 1. *cdc12* encodes a protein similar to the dia/BNI1 family of proteins. (*A*) Localization of the *cdc12* gene. Restriction map of the original 6-kb genomic clone insert (pFC103) that rescues *cdc12-112* mutant cells. (*Arrow*) Extent and orientation of the single large open reading frame in the region. Truncated versions of the clone and their ability to complement a *cdc12-112* mutant; ++, full rescue; +, partial rescue. *cdc12::ura4* deletion construct used in deleting *cdc12.* Much of the *cdc12* open reading frame is replaced with a 1.6-kb *ura4* insert. (*B*) *cdc12* open reading frame. Regions of predicted coiled-coil are underlined (Lupas et al., 1991), and the polyproline regions are boxed. (*C*) Similarity in gene domain structure. Proteins share a central FH1 polyproline rich domain (*black box*), an FH2 domain (*stripe box*), and a larger COOH-terminal area of homology (*gray*) also diagrammed in *D.* (*D*) Similarity of the proteins in a COOH-terminal region. Proteins were aligned using Megalign in DNA Star. Residues identical to a calculated consensus are colored in black, and conserved residues are colored in gray. These sequence data are available from GenBank/EMBL/DDBJ under accession number Q10059.

containing appropriate dilutions of protein, and 140 μl of CB buffer (0.6 M sorbitol, 50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 0.06% Triton X-100) were mixed and incubated in a 200 μl tube at room temperature with inversion for 1 h. In PLP inhibition experiments, 5 μl PLP or buffer alone was added to the binding reactions. Aliquots were taken for total sample. Supernatant and pellet fractions were collected upon centrifugation in a picofuge (Stratagene) for 30 s. Fractions were analyzed by SDS-PAGE gels and Coomassie staining. Densitometry of bands was performed with an IS-1000 gel documentation system (Alpha Innotech Corp., San Leandro, CA).

Results

cdc12 Encodes a Member of a Newly Recognized Family of Cytokinesis Proteins

The fission yeast *cdc12-112* mutant is a recessive temperature-sensitive lethal mutant with a specific defect in actin ring formation and cell division (Nurse et al., 1976; Marks et al., 1987; Chang et al., 1996). We cloned the *cdc12* gene by screening for a plasmid from a genomic DNA library that could rescue the temperature-sensitive lethality of *cdc12-112* mutant. Multiple genetic criteria showed that the plasmid pFC103 contained the *cdc12* gene (see Materials and Methods). Sequence analysis of the clone revealed one large open reading frame (Fig. 1 *A*). Deletion analysis (Fig. 1 *A*) showed this open reading frame is responsible for complementation of the *cdc12-112* mutation. Comparison of nucleotide sequences from genomic and cDNA clones helped to delineate the ends of the mRNA transcript and demonstrated that *cdc12* does not contain introns.

Conceptual translation of the nucleotide sequence predicts that *cdc12* encodes a polypeptide of 1,841 amino acid residues. Features in the deduced polypeptide include two predicted coiled-coil domains (Lupas et al., 1991) and a central region containing two stretches of polyproline (Fig. 1 *B*). Database searches using the BLAST algorithm revealed that the *cdc12* gene product is similar to an emerging family of proteins that include Bni1p from *S. cerevisiae*, diaphanous from *Drosophila*, fus1p from *S. pombe*, figA/sepA from *Aspergillus nidulens*, cappuccino from *Drosophila*, and formins from mouse, human, and chicken (Fares, H., and J. Pringle, personal communication; Petersen et al., 1995; Harris et al., 1997; Castrillon and Wasserman, 1994; Emmons et al., 1995; Woychik et al., 1990; Maas et al., 1991; Trumpp et al., 1992). Of these proteins, diaphanous, Bni1p, sepA, and cappuccino have been implicated in cytokinesis (Castrillon and Wasserman, 1994; Kohno et al., 1996; Harris et al., 1997; Manseau et al., 1996).

These proteins share several regions of homology, including two domains that have been termed FH1 and FH2 (formin homology domains) (Castrillon and Wasserman, 1994). The FH1 domain includes proline-rich sequences

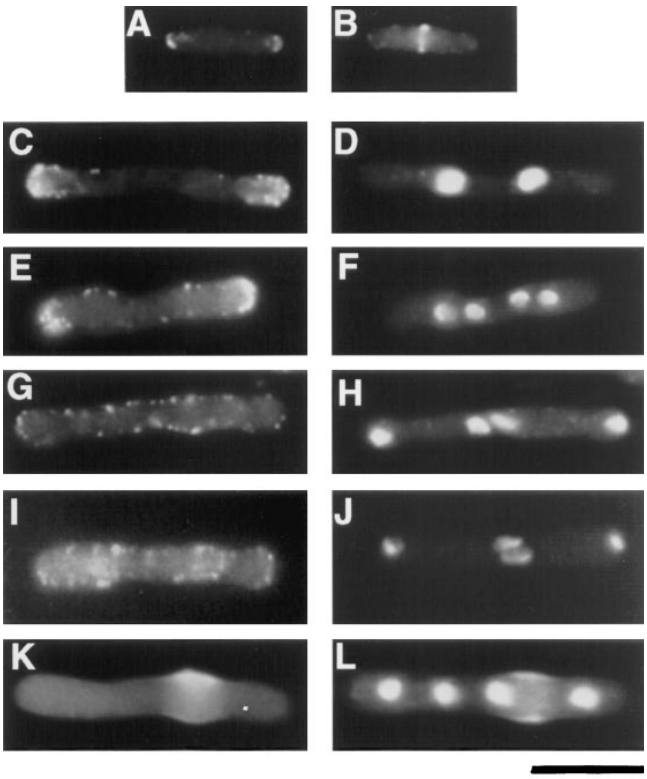

Figure 2. Phenotype of *cdc12*$^+$ and *cdc12::ura4* deletion cells. (*A* and *B*). Wild-type cells stained with rhodamine-phalloidin, showing normal interphase (*A*) and mitotic (*B*) actin patterns. (*C–L*) *cdc12::ura4* cells stained with rhodamine-phallodin (*C*, *E*, *G*, and *I*), DAPI (*D*, *F*, *H*, and *J*), or calcofluor (*K* and *L*). Diploid *ura4/ura4 leu1/leu1 ade6-M16/ade6-M22 cdc12*$^+$*/cdc12::ura4* cells were sporulated, and the spores were germinated in media containing no uracil, so only *cdc12::ura4* spores could germinate. Spores were grown for 15 h at 30°C, fixed, and stained. (*C–F*) *cdc12::ura4* cells in interphase, showing normal interphase polarized actin distribution at the cell tips. Cell in *D* has two nuclei, and cell in *F* has proceeded through another generation and contains four nuclei. (*G–J*) *cdc12::ura4* mitotic cells with actin dots instead of an actin ring. Cell in *G* is focused on the middle of the cell, while the cell in *I* is focused on the cell surface. Nuclear patterns in *H* and *I* are characteristic of cells in mitosis (see Fig. 6 *A* for comparison). Calcofluor-stained cells show no septum cell wall deposition even after multiple nuclear divisions. The rounded bulge that stains with Calcofluor (*K* and *L*) in *cdc12::ura4* cells represents the site of the original spore wall. Bar, 10 μm.

that are potential binding sites for profilin and for SH3 domains (Tanaka and Shibata, 1995; Mayer and Ekd, 1995). The FH2 domain is a conserved region (*striped box* in Fig. 1 *C*) located near the COOH terminus. We also detected an additional 300–amino acid region of similarity in the COOH-terminal part of these proteins encompassing the FH2 domain (see *gray box* in Fig 1 *C*; Fig. 1 *D*). The similarity is the strongest among the fungal members: cdc12p is 31–35% identical and 56–60% similar in this 300–amino acid domain to the other fungi proteins, and 26% identical and 50% similar over the whole proteins. Phylogenetic tree analysis shows *S. pombe* fus1 is the most similar to cdc12. cdc12p has little detectable similarity with the vertebrate formins outside of the FH1 and FH2 regions. cdc12p, fus1p, Bni1p, and figAp also share limited regions of homology in the NH_2-terminal half of the proteins (not shown). Deletion analysis of *cdc12* suggests that the COOH-terminal two-thirds of the protein, which contains the conserved FH1 and FH2 domains, is essential and sufficient for activity (Fig. 1 *A*).

cdc12 Is an Essential Gene Required for Actin Ring and Septum Formation

To determine the function of *cdc12*, we examined the effects of a *cdc12* deletion. A *cdc12::ura4* deletion construct was made in which >70% of the open reading frame, including the conserved FH1 and FH2 domains, was replaced by the *ura4* gene. This construct was transformed into a diploid strain, and multiple stable ura$^+$ diploid transformants, which were confirmed to be heterozygous for a homologous replacement of the *cdc12* gene, were sporulated and analyzed. Tetrad analysis showed that at least two of the four spores were dead and that there were no viable haploid *ura4*$^+$ spores, demonstrating that the *cdc12::ura4* haploid cells were not viable. Microscopic examination of individual spores showed that two inviable spores of each tetrad germinated and grew into a single large swollen dumbbell- or pear-shaped cell that did not divide. In random spore analysis, introduction of a plasmid containing the *cdc12* gene rescued the lethality of the *cdc12::ura4* spores, indicating that the lethal phenotype associated with the *cdc12::ura4* was due to the disruption of the *cdc12* gene. Thus, *cdc12* is essential for viability.

Germinated *cdc12::ura4* spores were examined for effects on actin and septum organization. Wild-type cells form cortical actin patches at the ends of cells during interphase and an actin ring during mitosis (Fig. 2, *A* and *B*; Marks and Hyams, 1985). *cdc12::ura4* cells had normal interphase cortical actin patches (Fig. 2, *C* and *E*), but only delocalized actin patches instead of an actin ring during mitosis (Fig. 2, *G* and *I*). In some mitotic cells, actin dots were concentrated in the zones where the actin ring would have formed (Fig. 2 *I*). *cdc12::ura4* cells became multinucleate (Fig. 2), indicating that nuclear division occurred in the absence of cytokinesis. This phenotype is identical to that seen in temperature-sensitive *cdc12* alleles at restrictive temperature (Chang et al., 1996; Marks et al., 1987; Nurse et al., 1976). Thus, *cdc12* is required for actin ring assembly during mitosis, but is not required for actin organization during interphase.

cdc12::ura4 cells had no depositions of septal material (Fig. 2, *K* and *L*). This is in contrast to all known temperature-sensitive alleles of *cdc12*, which display multiple aberrant patches of septal materials (Chang et al., 1996; Streiblova, 1984; Nurse et al., 1976). Thus, *cdc12* is also necessary for septum formation.

cdc12 Is a Component of the Cell Division Ring

To localize the *cdc12* gene product, we raised polyclonal rabbit antibodies against a cdc12 FH1 domain protein fragment expressed in *E. coli.* The affinity-purified antibody recognized on an immunoblot a single band of ~220 kD (Fig. 3 *A*), which is consistent with the size predicted from the nucleotide sequence. This 220-kD protein was confirmed to be cdc12p by showing that the level of this protein is increased ~10–20-fold in cells containing a plasmid

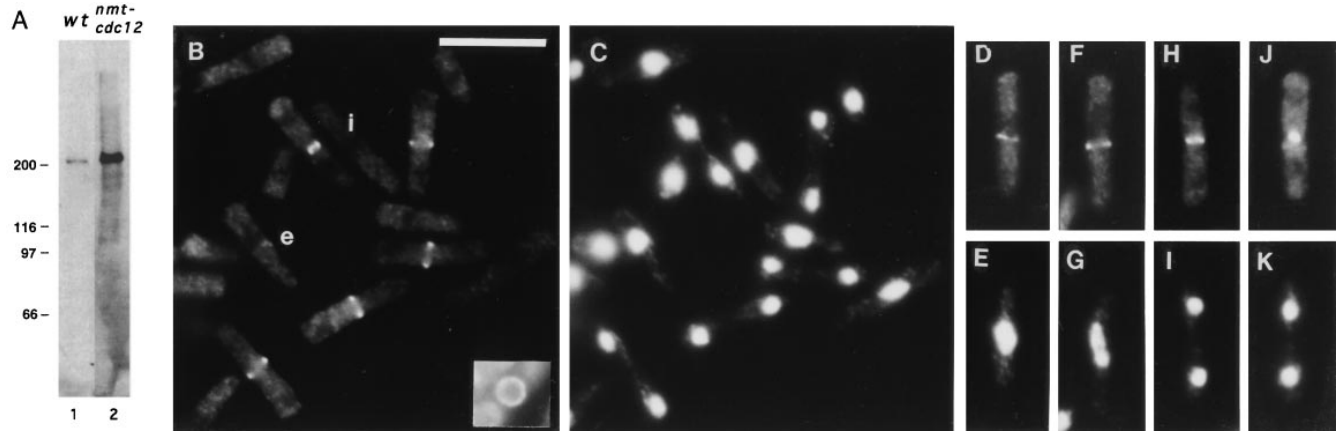

Figure 3. cdc12p localizes to the cell division ring in wild-type cells. (*A*) Specificity of the anti-cdc12 affinity-purified antibody. 5 μg of crude whole yeast cell extracts were analyzed by Western blotting using affinity-purified anti-cdc12 antibody. (Lane *1*) Extract from wild-type cells. (Lane *2*) Extract from cells overexpressing cdc12p. Cells transformed with the plasmid pnmt-cdc12 (FC291) were shifted to derepressing conditions (minimal media without thiamine) for 20 h at 30°C. Molecular weight markers (kD) are indicated. Serial dilutions showed that the pnmt-cdc12 extract contains 10–20-fold more cdc12p than the wild-type extracts (data not shown). (*B–K*) cdc12p localization in wild-type cells. Wild-type cells were grown in YE5S at 30°C in exponential phase, fixed, and processed for immunofluorescence using anti-cdc12 antibody and DAPI (see Materials and Methods for details). (*B*, *D*, *F*, *H*, and *J*) Anti-cdc12 staining. (*C*, *E*, *G*, *I*, and *K*) DAPI staining. (*B* and *C*) A typical field of cells containing cells from different phases of the cell cycle. Note examples of ring staining. (*e*) Cell in early mitosis in which the ring is beginning to form in an asymmetrical fashion. (*i*) Interphase with no specific cdc12 staining. (*B*, *inset*) Cross-section of a cdc12p ring. (*D* and *E*) Cell in early mitosis; (*F* and *G*) cell in early anaphase; (*H* and *I*) cell in mid-anaphase; (*J* and *K*) cell in postanaphase during ring closure. Patchy cytoplasmic staining is also seen in cells lacking cdc12p, and thus probably represents background staining (data not shown). Bar, 10 μm.

that overexpresses cdc12p from the thiamine-regulated *nmt* promoter (Fig. 3 *A*). Measurements of cdc12p levels both from synchronized cells and from cells arrested in G2 (*cdc25* block) and G1 phases (*cdc10* block) showed that cdc12p is expressed throughout the cell cycle with no large fluctuations in protein levels or mobility shifts in different phases of the cell cycle (data not shown).

Immunofluorescence using this anti-cdc12p antibody revealed staining of the fission yeast cell division ring (Fig. 3). cdc12p was present in early mitotic cells as a thin, faint ring (Fig. 3 *D*). This ring was more intense during anaphase (Fig. 3 *H*) and, after completion of anaphase, appeared to contract to a single dot between the daughter cells during cytokinesis (Fig. 3 *J*). cdc12p staining was very similar to staining patterns of other components of the ring such as actin and cdc4p (Marks and Hyams, 1985; McCollum et al., 1995). cdc12p did not localize to other actin structures, such as interphase actin patches at the ends of cells or actin dots adjacent to the septum during septation (Marks and Hyams, 1985). The cdc12p staining was specific because there was no ring staining in *cdc12-112* mutant cells (see Fig. 5 *A*), and, in addition, a green fluorescent protein–cdc12 fusion is located at similar structures (Chang, F., unpublished observations).

In interphase cells, only weak, diffuse cytoplasmic staining was detected (Fig. 3 *B*, see cell *i*). This may be largely nonspecific background staining, since similar weak cytoplasmic staining was also present in *cdc12-112* (see Fig. 5 *A*) and *cdc12::ura4* cells (data not shown).

Cells Overexpressing cdc12 Exhibit a cdc12p Spot

We also examined cdc12p localization in cells overexpressing cdc12p to attempt to visualize cdc12p structures that may be too small or stained too weakly in wild-type cells. A moderate strength thiamine-repressible *nmt1* promoter (derived from pREP41x) was cloned 5′ of the *cdc12* open reading frame (pnmt-cdc12; see Materials and Methods), and the construct was integrated in the chromosome. Cells carrying this construct expressed cdc12p at 10–20-fold higher than wild-type levels, but retained normal growth rate, cell morphology, cell division, and actin distribution after 20 h of induction (data not shown).

Overexpression of cdc12p revealed structures that were not previously observed in wild-type cells (Figs. 4 and 5; Table II). During interphase, the most common pattern (37% of the cells) was a discrete medial spot located between the nucleus and the cortex (Fig. 4, cell *1*; Table II). This dot was not associated with a similar dot of F-actin (data not shown). 18% of the cells exhibited a dot on the cell surface near the end of the cell, which is presumably a midbody remnant from a previous division (Fig. 4, cell *5*; Table II). 33% of the cells exhibited no specific staining, and other less frequent patterns such as multiple dots were also observed (Fig. 4, cell *6*; Table II). Medial spot staining appeared to be cell cycle regulated, as almost all (96%) cells at the very beginning of interphase (very short cells) exhibited no medial spot staining, while most (76%) late interphase cells (long cells) exhibited a medial spot.

In early mitosis, cells exhibited a similar medial spot (17%; Fig. 5 *C*), a spot with an intersecting faint cdc12 ring and strand (31%; Fig 5, *B* and *C*), or a ring similar to a wild-type cell (50%; Fig. 5 *D*). Examination of cells in different focal planes (Fig. 5) showed that the cdc12p spot is not, for instance, a ring seen in a particular focal plane (compare spot in cell *A* with ring in cell *D*). In cells where both a spot and a ring (or strand) are observed (Fig. 5, *B* and *C*), the two structures always coincided, suggesting

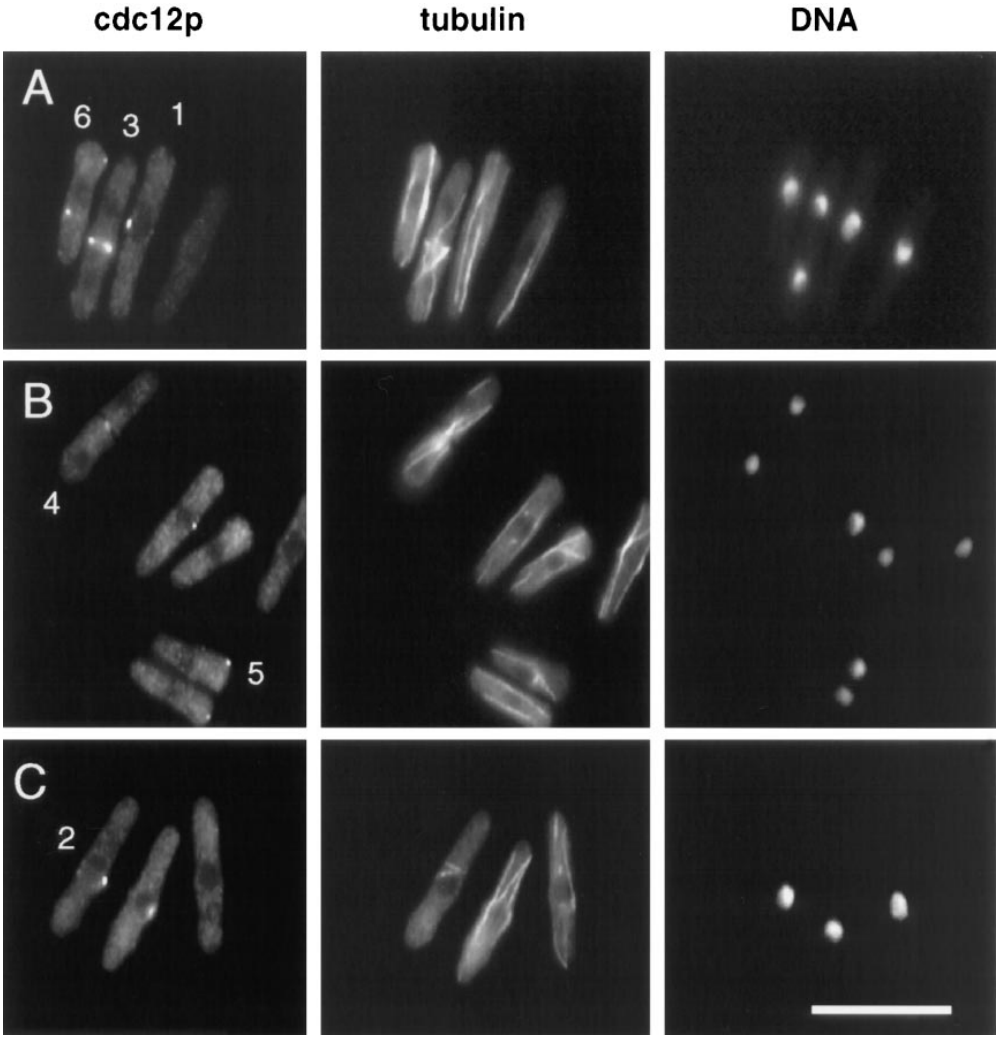

Figure 4. Upon overexpression, cdc12p localizes to a medial spot. FC565 (pnmt-cdc12) cells were induced in derepressing conditions for 20 h at 30°C, fixed in cold methanol, and processed for immunofluorescence with anti-cdc12 antibody (*left panels*), anti-tubulin antibody (*middle panels*), and DAPI (*right panels*). The tubulin and DAPI staining patterns identify the cell cycle stage. (*A–C*) All panels show three representative fields from the same population of cells. (Cell *1*) Interphase cell with a medial cdc12p spot. (Cell *2*) Early mitotic cell with a medial spot. (Cell *3*) Cell in postanaphase with a cdc12p ring. (Cell *4*) Cell in postanaphase with a closed ring. (Cell *5*) Cell after cell division with a cdc12p spot at the presumed midbody on the cell tip. (Cell *6*) Cell with two spots, one at the cell tip and one at the cell middle. Bar, 10 μm.

that the spot may mark or give rise to the ring. In these cells, about half of the rings appeared discontinuous around one side of the spot (Fig. 5 *C*, *top panel*). In late mitosis, nearly all cells exhibited a cdc12p ring as seen in wild-type cells (Fig. 4, cells *3* and *4*).

A corresponding medial spot was not readily detectable in wild-type cells that do not overexpress *cdc12*, and thus it is not known if cdc12p resides in such a spot in wild-type cells. A small faint dot in the proper location was occasionally seen, but it was usually not significantly more intense than other background cytoplasmic dots. However, we have observed cells in which cdc12p ring staining appears stronger at one side of the cell (see cell *e* in Fig. 3 *B*). Several explanations for the cdc12p spot are considered in the Discussion.

cdc12p Localization in Different Actin Ring Mutants

Next, we examined expression and localization of cdc12p in the different actin ring mutants. First, immunoblot analysis showed that expression of cdc12p was not altered in *cdc3*, *cdc4*, *cdc8*, *cdc15*, or *mid1* mutants (data not shown). Second, we examined cdc12p localization in different mutant backgrounds. *cdc3-313*, *cdc8-346*, and *cdc15-287* mutants generally showed no specific cdc12p staining during mitosis, much like a *cdc12-299* mutant (Fig. 6, *A–D*, *left panels*). In addition, *cdc15* mutant cells that overexpressed cdc12p did not exhibit any cdc12p spot or ring staining in either interphase or mitotic cells (data not shown). Thus, *cdc3* (profilin homologue), *cdc8* (tropomyosin homologue), and *cdc15* genes are necessary for cdc12p localization at the ring, and at least *cdc15* is also required for cdc12p spot formation.

In contrast, cdc12p was localized to a single medial cortical spot in *cdc4-377* mutant cells during mitosis (Fig. 6 *E*; Table II). While most early mitotic cells (76%) exhibited no staining, most late mitotic cells (76%) exhibited this spot (Table II), suggesting that this spot is formed during mitosis. In addition, some cells (6% of late mitotic cells) possessed a spot with a faint intersecting ring. Thus in a *cdc4* mutant, cdc12p accumulated in a medial spot in place of the ring. The relationship of cdc12p and actin was examined in *cdc4-377* mutant cells. Instead of an actin ring, these cells form an asterlike array of actin cables organized around a single cortical point (Fig. 6 *F*; Chang et al., 1996). Double staining revealed that the cdc12p dot was located to the center of the larger actin aster (Fig. 6 *F*).

mid1-366 mutants exhibited highly variable patterns that reflect multiple defects in the spatial organization of the ring (Fig. 7; Table II). Generally, *mid1* mutants had a medial

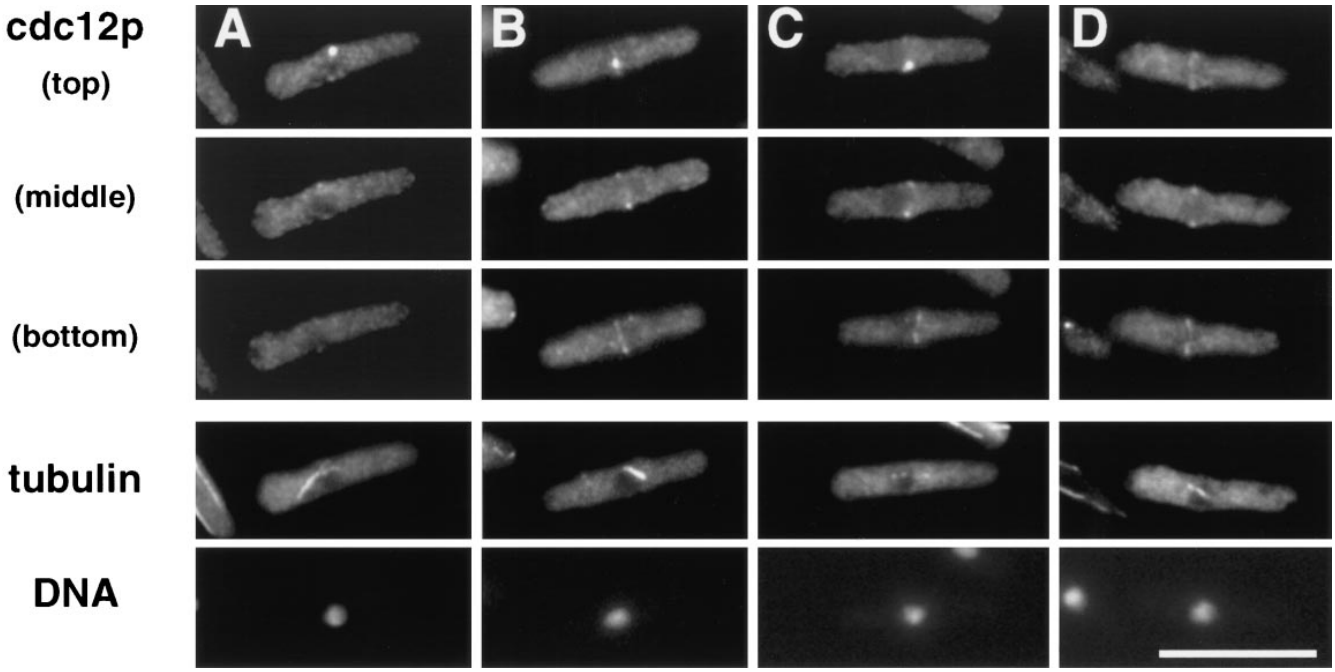

Figure 5. The positions of the cdc12p spot and the ring coincide. FC565 (pnmt-cdc12) cells were processed as described in Fig. 4. Shown are four representative cells in early mitosis, around the period of ring formation. Top three panels show three different focal planes of the same cell, and the bottom two panels show tubulin and DAPI staining as labeled. (*A*) Cell with a cdc12p spot. (*B*) Cell with a cdc12p associated with a complete ring. (*C*) Cell with a cdc12p spot with an incomplete ring. Note the discontinuity of the ring adjacent to the spot in the top panel. (*D*) Cell with a cdc12p ring but no spot. Bar, 10 μm.

cdc12p spot associated with single strand (Fig. 7, *A–C*). Over 90% of the strands were associated with the nucleus at some point along the strand, and some strands even wrapped around the nucleus (Fig. 7 *C*). Some cells only had a medial spot of cdc12p (Fig. 7 *D*) or no staining at all (not shown). 30% of the spots were positioned away from the cell middle and the nucleus (Fig. 7 *E*), indicating an apparent defect in placing the spot. Although few (<5%) com-

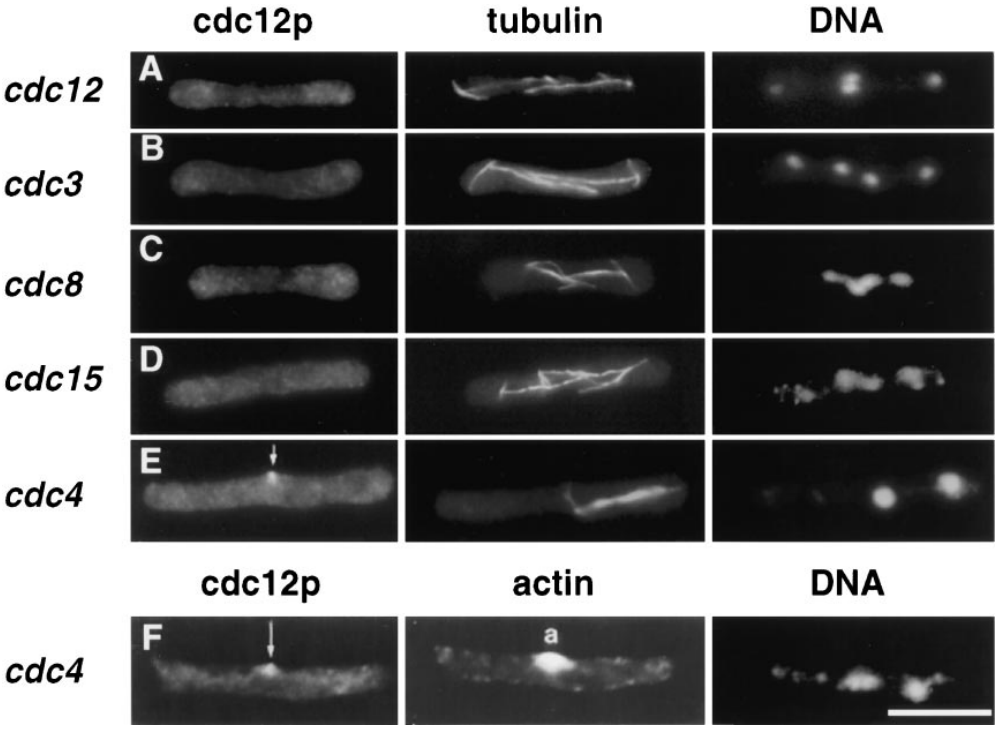

Figure 6. Localization of cdc12p in different actin ring mutants during mitosis. (*A*) *cdc12-299* (FC127); (*B*) *cdc3-313* (FC114); (*C*) *cdc8-346* (FC131); (*D*) *cdc15-287* (FC55); (*E* and *F*) *cdc4-377* (FC361). Mutant cells were grown in YE5S at permissive temperature (25°C), and then shifted to restrictive temperature (35.5°C) for 4 h. Cells were fixed and processed for immunofluorescence with anti-cdc12 antibody, anti-tubulin antibody or anti-actin antibody, and DAPI. On the basis of spindle and nuclear morphology, cells pictured are in anaphase, the cell cycle period of maximum cdc12p ring staining in wild-type cells. (*A–C*) Cells are in mitosis in which two nuclei are dividing into four and thus exhibit double spindles; (*E–F*) cells exhibit single spindles. (*A–C*) Mutants exhibit no specific cdc12p staining; the cytoplasmic staining is nonspecific background staining. (*E* and *F*) Note the medial spot of cdc12p (*arrow*) in the *cdc4* mutant, which localizes to the origin of an actin aster (*F*, *middle panel*, labeled *a*). Bar, 10 μm.

Table II. cdc12p Localization Patterns in Wild-type and Mutant Cells

Cell type	Number of cells assayed	Percentage of cells with particular cdc12p localization pattern (a b c d e f g h)							
		%							
Wild type interphase	300	100	0	0	0	0	0	0	0
Wild type early mitosis	50	52	0	0	48	0	0	0	0
Wild type late mitosis	80	2	0	0	98	0	0	0	0
OP *cdc12* interphase	205	33	37	0	0	18	12	0	0
OP *cdc12* early mitosis	70	1	17	31	50	0	0	0	0
OP *cdc12* late mitosis	80	1	0	0	99	0	0	0	0
cdc8 mitosis	50	100	0	0	0	0	0	0	0
cdc15 mitosis	50	88	0	0	6	0	0	0	0
cdc4 early mitosis	46	76	13	0	11	0	0	0	0
cdc4 late mitosis	46	15	76	6	2	0	0	0	0
mid1 early mitosis	70	39	16[i]	0	0	0	0	3	43
mid1 late mitosis	70	4	3	0	13	0	0	34	46

Cells were fixed and stained with anti-cdc12 and anti-tubulin antibodies and DAPI. Individual cells were first screened by fluorescence microscopy for a particular cell cycle stage on the basis of tubulin staining, and were then examined for cdc12p localization. Cells with a short mitotic spindle before nuclear division were designated as being in early mitosis. Cells in anaphase or postanaphase were designated as being in late mitosis. Cells with an interphase microtubule array and a single nucleus were designated as being in interphase. Dots and lines indicate cdc12p localization, and the empty circle indicates the nucleus. OP (overproducing) *cdc12*: cells (FC566) that contain a chromosomal integrated copy of pnmt-cdc12 were induced in minimal media for 20 h at 30°C. *cdc8-346* (FC131), *cdc15-287* (FC55), and *cdc4-377* (FC361) mutants were grown for 4 h at the restrictive temperature of 35.5°C. *mid1-366* (FC164) cells were grown for 2 h at 35.5°C. (*a*) Cells with no specific cdc12p staining. (*b*) Cells with a medial cdc12p spot. Interphase spots were adjacent to the nucleus. (*c*) Cells with a cdc12p spot with an intersecting ring. (*d*) Cells with a normal cdc12p ring. (*e*) Cells with a cdc12p dot near the end of the cell, which probably is a midbody remnant, left from a previous division. (*f*) Cells with multiple dots in overexpressing cells or dots not covered in *b* or *e*. Many cells in this category have a combination of patterns seen in *b* and *e*. (*g*) Cells with an intact but displaced ring. (*h*) Cells with a cdc12p strand. Many are associated with a dot, and >90% are associated with the nucleus. (*i*) In *mid1* cells, dots are not always on the cortex, and some dots (~30%) are displaced away from the medial nucleus.

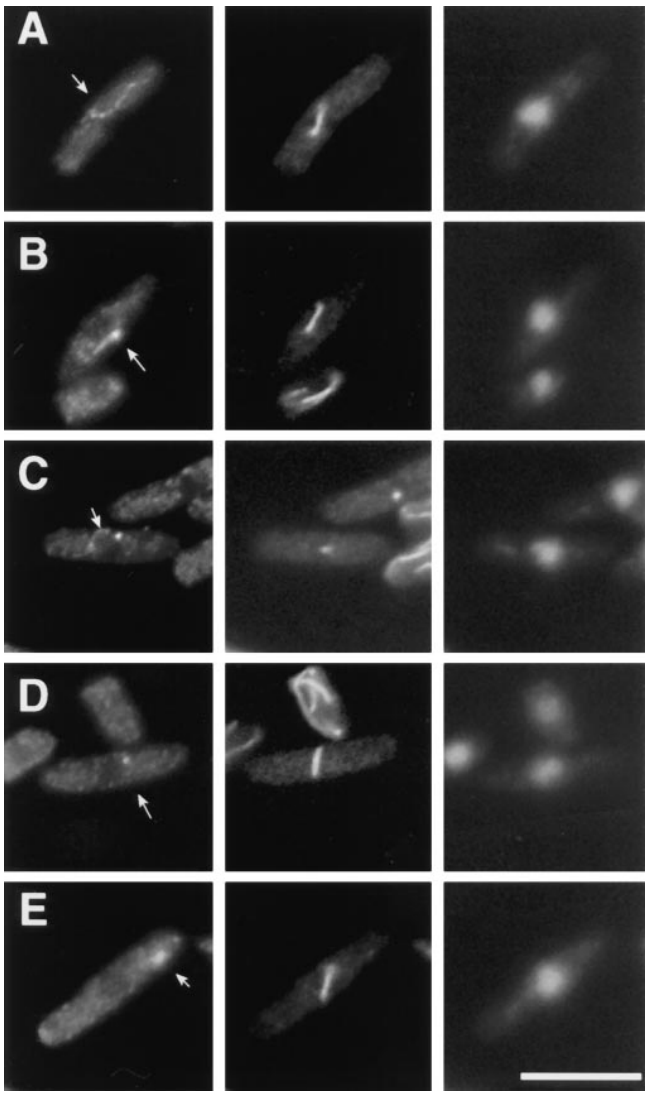

Figure 7. Localization of cdc12p in *mid1* mutant cells during early mitosis. *mid1-366* mutant cells were grown in YE5S at permissive temperature (25°C), and then shifted to restrictive temperature (35.5°C) for 2 h. Cells were processed for immunofluorescence with anti-cdc12 antibody, anti-tubulin antibody or anti-actin antibody, and DAPI. On the basis of spindle and nuclear morphology, cells pictured are in early mitosis, the cell cycle period around ring formation. Note different configurations of cdc12p dot and strand (*arrows*). Bar, 10 μm.

plete rings were present in early mitosis, many complete rings that were displaced and tilted (47% of cells) were seen in late mitosis (Table II; see Chang et al., 1995), suggesting that the cdc12p strand in early mitosis may organize into a displaced ring later in mitosis. Thus, *mid1* mutants have defects both in guiding a strand around the circumference of the cell to form a ring and in the medial placement of the spot.

Evidence That cdc12p Interacts with Profilin cdc3p

The existence of proline-rich sequences in cdc12p suggests that cdc12p may bind to the profilin homologue cdc3p, since profilins are known to bind to proline-rich sequences (Tanaka and Shibata, 1985). In addition, evidence that *cdc3* and *cdc12* share similar mutant phenotypes (Chang et al., 1996) and that both gene products localize in the septum region during cell division (Balasubramanian et al., 1994; this work) suggests that these proteins may interact in vivo. Therefore, we tested for the interaction between these gene products using genetic and biochemical methods.

First, we tested whether *cdc3* and *cdc12* interact genetically by constructing *cdc12* and *cdc3* double mutants. Fig. 8 shows the phenotypes of wild-type, *cdc3*, *cdc12*, and *cdc3 cdc12* mutants. The *cdc3-313 cdc12-112* double mutants had a much more severe phenotype than either single mutant. *cdc3 cdc12* mutant spores were either dead at 25°C or produced cells with very poor growth at 25°C and no growth at the intermediate temperature of 29°C. In comparison, single *cdc3* and *cdc12* mutants grew well at 25°C and 29°C. The single mutants and the double mutants did not grow at 36°C. Thus, *cdc3* and *cdc12* genes exhibit a

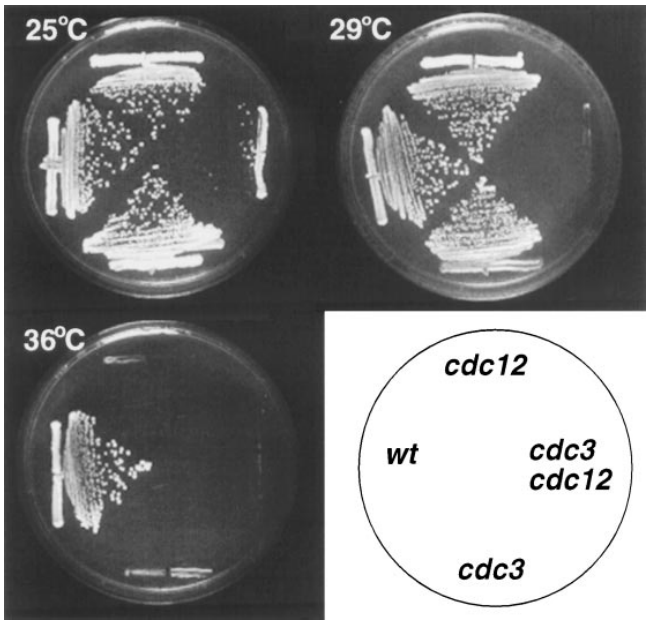

Figure 8. *cdc3* and *cdc12* exhibit a synthetic-lethal genetic interaction. Siblings from a tetratype tetrad in a cross between *cdc3-313* and *cdc12-112* mutants were streaked on YE5S plates and incubated at 25°, 29°, or 36°C. Growth was assayed by colony formation. Note that the *cdc3 cdc12* double mutant exhibited markedly reduced growth at 25° and 29°C where the single mutants are viable. Strains were FC322 (*cdc12-112*), FC323 (*wt*), FC324 (*cdc3-313*), and FC325 (*cdc3-313 cdc12-112*).

synthetic-lethal interaction, which suggests that these gene products may interact at some level in vivo.

Next, we tested whether cdc3p and cdc12p interact directly by an in vitro binding assay. GST–cdc3p fusion protein and GST protein were expressed in *E. coli* and purified on glutathione-coupled agarose beads (Fig. 9 *A*, lanes *1* and *2*). As a full-length cdc12 fusion protein was not expressed well in *E. coli*, a smaller His_6-tagged fragment of cdc12p that included the central proline-rich FH1 domain was expressed in *E. coli* and purified using Ni-affinity chromatography (Fig. 9 *A*, lane *3*). The cdc12p protein fragment was mixed with GST-cdc3 beads or GST beads, and binding was assayed by determining the amount of cdc12p that pelleted with the beads. Fig. 9, *B* and *C*, show that cdc12p bound to beads containing GST-cdc3 but did not bind to beads with GST or glutathione alone. This cdc12p binding was dependent on the concentration of cdc3p. To test if the proline-rich region of cdc12p might be responsible for profilin binding, we assayed for binding in the presence of poly-L-proline competitor. Binding was inhibited by the addition of PLP peptides (Fig. 9, *D* and *E*). A 5:1-fold molar excess of the PLP peptide to cdc12 was sufficient for inhibition, while a 1:2 molar deficiency was not, suggesting that this inhibition is specific. Addition of 1% BSA did not abolish binding (data not shown). Further experiments will be necessary to derive an accurate binding constant. These data indicate that the cdc12p fragment is capable of binding to cdc3p profilin in a direct and specific manner and that the interaction is likely to be mediated by the PLP binding site in profilin- and proline-rich sequence (s) present in the FH1 domain of cdc12p.

Discussion

cdc12p and Cell Division

cdc12 encodes a member of an emerging family of proteins that function in cytokinesis or other actin-mediated processes. The mutant phenotype of *cdc12* indicates that it is required for actin ring formation and septum formation in cell division: *cdc12* mutants exhibit actin dots instead of an actin ring, do not form a septum, and accumulate as large, multinucleate cells. cdc12p localization at the ring indicates that it is likely to be a structural component of the cell division ring. In contrast with some actin-binding proteins that organize multiple actin structures (Balasubramanian et al., 1992), *cdc12* may function specifically in ring formation during mitosis: *cdc12* mutants are not affected in actin patch organization during interphase (Chang et al., 1996; Fig. 2), and cdc12p is not located with other actin structures such as actin patches. cdc12p also has a role in directing septum cell wall formation outside the plasma membrane proximal to the ring and may be involved in targeting cell wall synthetases to the ring site.

Interaction between cdc12p and Profilin

We imagine that cdc12p is a component of a multiprotein complex that organizes actin at the plasma membrane. Here we have found evidence that cdc12p binds directly to the actin-binding protein, profilin cdc3p. Since profilin will bind to eight to ten sequential prolines (Machesky and Pollard, 1993; Perelroizen et al., 1994), it is likely that the sequential stretches of nine and six prolines in the FH1 domain of cdc12 contribute to this interaction.

Four in vivo observations support the in vitro interaction data. First, *cdc3* and *cdc12* mutants have very similar phenotypes: both mutants are defective in actin ring assembly and display disorganized actin dots in place of a ring during mitosis (Chang et al., 1996; Marks et al., 1985; Balasubramanian et al., 1994). Second, cdc3p and cdc12p are located in the same regions of the cell: cdc3 is located during mitosis in a band at the cell middle that overlaps but is broader than the narrow ring of cdc12p (Balasubramanian et al., 1994). Third, *cdc3* is required for the localization of cdc12p at the ring, since cdc12p is not localized in a *cdc3* mutant (Fig. 6 *B*). Fourth, *cdc3* and *cdc12* mutants exhibit a synthetic-lethal genetic interaction. Together, the genetic and biochemical data support the idea that cdc12p and profilin interact physically and function together in actin ring assembly.

Though profilin has been intensely studied, its role in regulating actin in vivo is still not well understood. In vitro, profilin has the potential both to inhibit actin polymerization by binding to actin monomers as well as to drive actin polymerization, possibly by affecting the nucleotide-binding state of actin and by lowering the critical concentration for actin polymerization (Pantaloni and Carlier, 1993; Theriot and Mitchison, 1993). In vivo, profilin is often located at sites of active actin assembly, for instance, at the end of *Listeria* motile bacterium, where it may promote actin polymerization (Theriot et al., 1994). In *S. pombe*, profilin appears to have an active role in actin ring assembly (Balasubramanian et al., 1994). Therefore, cdc12p may influ-

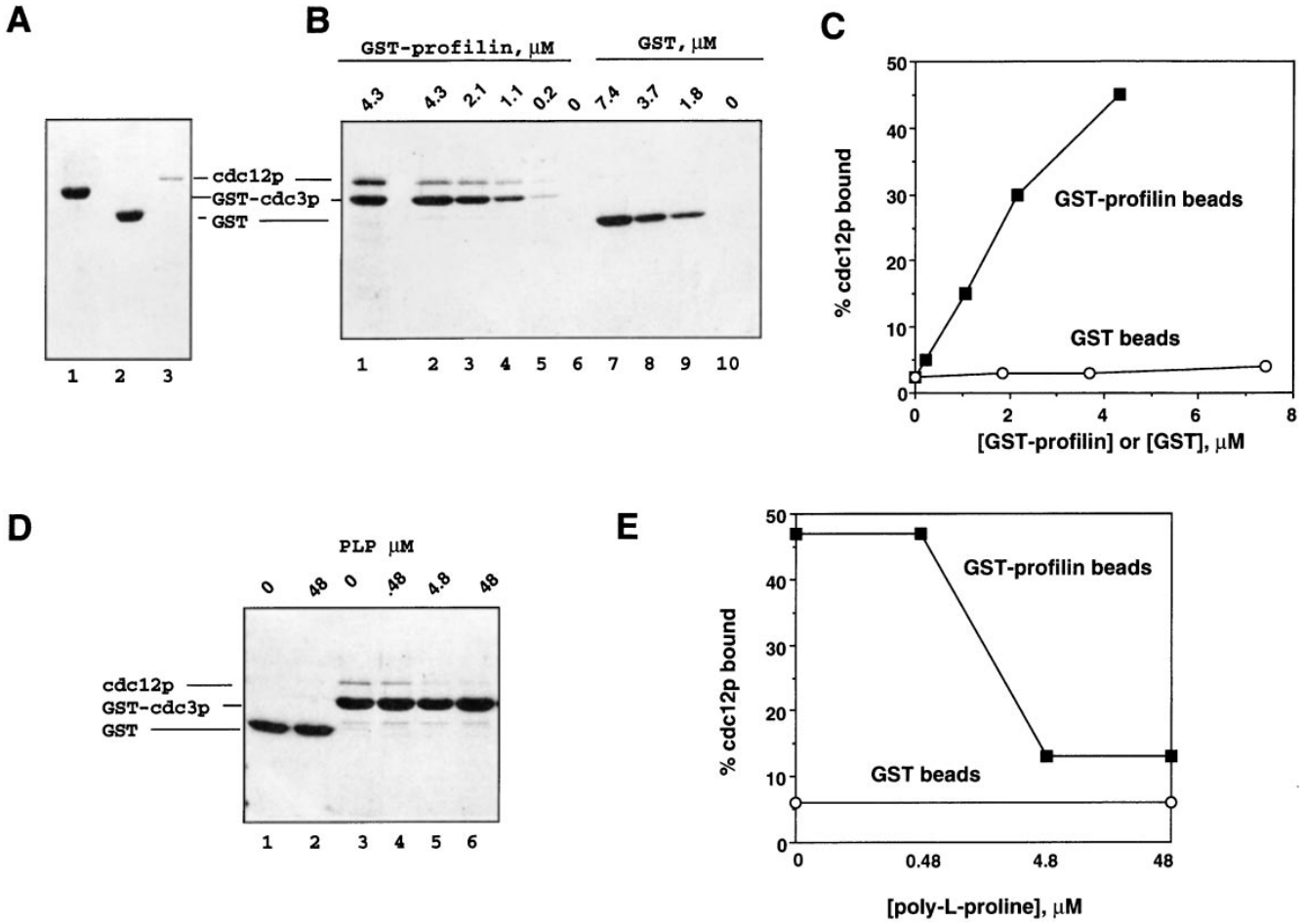

Figure 9. cdc12p fragment binds to profilin cdc3p in vitro. (*A*) Purified proteins used in these studies. (Lane *1*) Purified GST-cdc3. (Lane *2*) Purified GST. (Lane *3*) Purified His-tagged cdc12p fragment. (*B* and *C*) cdc12p binds to GST-cdc3 but not to GST. In each 180-μl reaction, cdc12p (1.6 μM final concentration) and 20-μl 50% slurries of glutathione agarose beads containing indicated concentrations of GST-cdc3 or GST were added to each reaction. Reactions were incubated at room temperature for 1 h, and the beads were pelleted. (*B*) Coomassie-stained gel of total proteins before pelleting (lane *1*) and pellet fractions (lanes *2–10*) at equivalent volumes. Bands were quantitated by densitometry relative to total protein in lane *1* and plotted in *C*. (*D* and *E*) Poly-L-proline inhibits binding. Varying amounts of poly-L-proline were added to the binding reactions similar to ones described above except cdc12p was 0.8 μM. (*D*) Coomassie-stained gel of the pellet fractions. (*E*) cdc12p in the pellets was quantitated by densitometry relative to supernatant fractions (not shown).

ence actin assembly during ring formation through this interaction with profilin.

There is evidence that other members of the cdc12p family also interact with profilin. In *Drosophila*, cappuccino and profilin mutants possess similar phenotypes in oogenesis, and an interaction has been demonstrated in the two hybrid system (Manseau et al., 1996). Two-hybrid, genetic, and biochemical data also suggest that *S. cerevisiae* BNI1 interacts with profilin (Evangelista et al., 1997). Recently, two other proline-rich proteins have also been implicated as profilin ligands: VASP and Mena are related proteins that appear to regulate actin assembly at focal adhesions and at the actin tail of the motile bacteria *Listeria* (Reinhard et al., 1992, 1995; Chakraborty et al., 1995; Gertler et al., 1996). Since profilin and VASP are associated with a nucleating center for the actin tail of *Listeria*, one possibility is that cdc12p and profilin are associated with a nucleating center for the ring.

cdc12p may possess additional binding partners. BNI1p has been found to interact with rho GTPases (Kohno et al., 1996; Evangelista et al., 1997). The cdc12p FH1 domain contains proline-rich consensus sequences for binding to SH3-binding domains. The two coiled-coil domains of cdc12p are potential sites for intramolecular or intermolecular interactions with other cdc12p protein molecules or with other coiled-coil proteins such as cdc15p, myosin, or septins. A large protein with multiple domains, cdc12p may act as a scaffold that brings together multiple ligands.

Evidence for a Pathway in Ring Formation

Analysis of cdc12p localization in different cell types revealed intriguing novel patterns that suggest that the site of ring assembly is initiated from a single point (Fig. 10). In cdc12p-overexpressing cells, the cdc12p was localized at a single medial spot between the nucleus and cortex during late interphase and early mitosis (Figs. 4, 5, and 7). This cdc12p spot was located precisely at the future site of

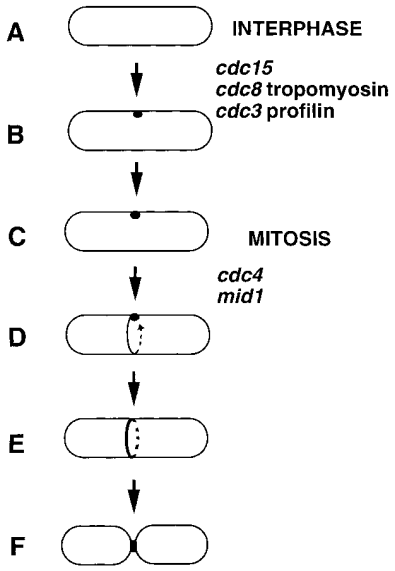

Figure 10. A model for the establishment of the division site in *S. pombe.* (*A* and *B*) In interphase, a marker spot is established at the cell middle, possibly by a signal from the nucleus. This spot may contain cdc12p; alternatively, upon overexpression, cdc12p may accumulate at such a structure. (*C*) In early mitosis, the spot remains on the cortex. (*D*) In early mitosis, the ring forms at the site marked by the spot by extension of a strand from the spot around the circumference of the cell. (*E*) During anaphase, the ring becomes more robust. (*F*) After nuclear division, the ring closes during septation and cell division. Roles of the actin ring genes are inferred from cdc12p localization in mutants. Aspects of this model are inferred from cdc12p overexpression and mutant data. Alternate models are discussed in the text.

ring assembly and generally did not include F-actin. It thus preceded ring assembly and appeared to mark the future division site.

A similar cdc12p spot was also observed in *cdc4* and *mid1* mutant cells. *cdc4* mutant cells exhibited a single spot instead of a ring during mitosis. *mid1* mutants often exhibited a single strand of cdc12p that appeared to emanate from a cdc12p spot on the nucleus in a seemingly random direction. In these cases, cdc12p is not overexpressed, but an aspect of ring assembly is defective.

Based upon these observations, we propose a speculative model for ring positioning and assembly in fission yeast (Fig. 10). During interphase and early mitosis, the cell middle may be marked by a single spot of cdc12p (Fig. 7, *A* and *B*). In mitosis, a ring may form from this spot by a single strand of cdc12p that originates from the spot and "migrates" around the circumference of the cell (Fig. 10 *D*). This strand migration model is consistent with patterns in *mid1* mutants, as described above, and in cells overexpressing cdc12p, in which incomplete rings that are discontinuous on one side of the spot can be observed (Fig. 5 *C*). This model is in contrast with an alternate de novo model in which the ring may arise de novo as a circumferential structure all along the medial axis. As the placement of the ring in fission yeast may be determined by a signal from the interphase nucleus (Chang and Nurse, 1996), the spot may represent the site where the nucleus signals the cortex.

Numerous aspects of this model remain to be tested. For instance, since a cdc12p spot was not readily visualized in wild-type cells without overexpression, it is not known if a cdc12p spot is present in interphase wild-type cells, or if cdc12p has an affinity for an as yet unidentified medial structure that does not contain cdc12p normally. An alternate model is that the cdc12p spots represent aberrant aggregates of cdc12p. For instance, overexpression of cdc12p during interphase may induce the formation of an aggregate of ring proteins including myosin that contracts into a single tight ball. However, in any of these models, the fact that the cdc12p spot anticipates the ring site suggests that spatial signals that position the ring are present during interphase and that cdc12p or associated proteins are capable of responding to these spatial cues. Thus, the cdc12p spot may be regarded as a marker that reveals the activity of spatial regulators that position the division site.

Roles of Ring Genes in Ring Formation

Analysis of different ring mutants suggests that the different ring genes play different roles in ring formation. *cdc12*, *cdc15*, *cdc3* (profilin homologue), and *cdc8* (tropomyosin homologue) appear to be involved in an early step in ring formation, possibly in nucleation of the ring. cdc12p is not localized in *cdc15*, *cdc3*, and *cdc8* mutants, suggesting that one role of these gene products is to localize cdc12p. cdc15p, an SH3 domain–containing protein, is an important regulator of ring formation in the cell cycle (Fankhauser et al., 1995) and is a candidate partner for binding an SH3-binding domain of cdc12p. cdc8p is a homologue of tropomyosin, a small actin-binding protein (Balasubramanian et al., 1992). Studies with the antiactin drug Latruculin A show that F-actin is also necessary for cdc12p localization (Engqvist, A., and F. Chang, unpublished observations). As proposed for bud site determination in *S. cerevisiae* (Yang et al., 1997), F-actin organized by actin-binding proteins may be required as a cortical scaffold that binds cdc12p at the cortex. At the cortex, cdc12p with profilin may then organize ring components, including F-actin, at the site. As demonstrated in Thyone sperm (Tilney et al., 1983), F-actin may also be used as a nucleation seed for cdc12p and profilin-dependent actin assembly. Finally, cdc12p and these ring components may be required all together for ring assembly, and loss of one of these essential components may cause delocalization of the other components.

In contrast, *cdc4* and *mid1* may not be necessary to localize cdc12p per se, but they may be required in a later step in ring formation. cdc4p is a putative myosin light chain, suggesting that a myosin-related protein is important in ring formation (McCollum et al., 1995). The *mid1* gene product is necessary for the spatial regulation of at least two aspects of ring formation: guiding a cdc12p strand around the circumference, and placing the initial spatial cue in the middle of the cell. *mid1* (*dmf1*) encodes a novel protein with a pleckstrin homology domain, PEST sequences, and a nuclear localization signal (Sohrmann et al., 1996). Intriguingly, the *mid1* gene product is located in the nucleus during interphase and in a medial ring in early mitosis, suggesting that it may link the positions of the nucleus and ring. However, our analysis shows that, in most cases, cdc12p

still appears associated with the nucleus in the earliest stages of ring formation in the *mid1-366* mutant.

Role of cdc12-like Proteins in Cytokinesis and Spatial Organization

cdc12p is member of a family of proteins involved in cytokinesis or other actin-related processes such as cell fusion. Although the overall amino acid sequence homology between these proteins is low (<30%), similarity in their domain structure, conservation of certain small domains such as the formin homology domains (Castrillon and Wasserman, 1994), and their roles in related cellular processes suggest that the function of these proteins is conserved. Many of these proteins are required in cytokinesis: like *cdc12*, *diaphanous* in *Drosophila* is necessary for cytokinesis (Castrillon and Wasserman, 1994), and *sepA*/*figA* in *Aspergillus* is required for septation (Harris et al., 1997). BNI1p in *S. cerevisiae* has a role in the placement of the division site (bud site placement in the bipolar pattern) (Zahner et al., 1996), displays genetic interaction with a septin, and is located at the division ring and at the site of polarized growth at the bud tip (Fares, H., M. Longtine, and J. Pringle, personal communication).

Other members of the cdc12p family have related roles in localizing other proteins or determinants at certain regions of the cell cortex. *cappuccino* is required both for cytokinesis and the establishment of polarity during *Drosophila* oogenesis and is potentially involved in the signaling events between the nucleus and cortex that establish polarity axes (Emmons et al., 1995; Roth et al., 1995). *cdc12*, *cdc3*, and *cdc8* may play analogous roles to cappuccino, profilin (chickadee), and tropomyosin II, which are required in the localization of posterior determinants in the *Drosophila* oocyte (Emmons et al., 1995; Erdelyi et al., 1995; Manseau et al., 1996). In *S. cerevisiae*, *BNI1* also is required for the proper localization of a determinant (Ash1p) in the daughter bud (Bobola et al., 1996). cdc12p is also similar to *S. pombe* fus1p, which is required for cell fusion and nuclear migration during mating and localized to patches at the projection tip (Petersen et al., 1995). *fus1* may be necessary to localize fusiogenic proteins to the projection tip during the mating process. Candidate vertebrate homologues of cdc12p are the formins, which have been implicated in limb morphogenesis (Woychik et al., 1990). However, the cellular role of the formins has not been determined. Further study of cdc12p and its homologues may help to elucidate signals that mark sites of cell division and polarity in many eukaryotes.

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