

Schizosaccharomyces pombe *cdc4*⁺ Gene Encodes a Novel EF-Hand Protein Essential for Cytokinesis

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Abstract. *Schizosaccharomyces pombe* cells divide by medial fission. One class of cell division mutants (*cdc*), the late septation mutants, defines four genes: *cdc3*, *cdc4*, *cdc8*, and *cdc12* (Nurse, P., P. Thuriaux, and K. Nasmyth, 1976. *Mol. & Gen. Genet.* 146:167–178). We have cloned and characterized the *cdc4* gene and show that the predicted gene product, Cdc4p, is a 141-amino acid polypeptide that is similar in sequence to EF-hand proteins including myosin light chains, calmodulin, and troponin C. Two temperature-sensitive lethal alleles, *cdc4-8* and *cdc4-31*, accumulate multiple nuclei and multiple improper F-actin rings and septa but fail to

complete cytokinesis. Deletion of *cdc4* also results in a lethal terminal phenotype characterized by multinucleate, elongated cells that fail to complete cytokinesis. Sequence comparisons suggest that Cdc4p may be a member of a new class of EF-hand proteins. Cdc4p localizes to a ringlike structure in the medial region of cells undergoing cytokinesis. Thus, Cdc4p appears to be an essential component of the F-actin contractile ring. We find that Cdc4 protein forms a complex with a 200-kD protein which can be cross-linked to UTP, a property common to myosin heavy chains. Together these results suggest that Cdc4p may be a novel myosin light chain.

IT has been proposed that the force required to constrict the cell circumference during cytokinesis is provided by the contractile ring, a transient structure containing F-actin that has been observed in diverse eukaryotic cell types (Satterwhite and Pollard, 1992). Myosin has also been found to be a component of the contractile ring (Schroeder, 1973; Fujiwara and Pollard, 1976; Yumura et al., 1984), and myosin motor activity is presumed to generate the forces necessary for contraction of the ring (Mabuchi and Okuno, 1977; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). In addition to actin and myosin, at least 14 other proteins have been found to localize to the contractile ring or cleavage furrow (reviewed in Satterwhite and Pollard, 1992; Balasubramanian et al., 1992, 1994; Neufeld and Rubin, 1994). Despite the large number of proteins implicated in cytokinesis, functional analysis of their roles has been limited.

Previous studies have suggested the value of a genetic approach to the study of cytokinesis. It has been shown

that inactivation of the *Dictyostelium discoideum* myosin heavy chain (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987) or either of its associated light chains (Pollenz et al., 1992; Chen et al., 1994) leads to a block in cytokinesis. In *Drosophila melanogaster*, genes essential for cytokinesis have also been identified in genetic studies (Schweigsuth et al., 1990; Karess et al., 1991; Neufeld and Rubin, 1994). Yeasts offer certain advantages over these other systems, and *Schizosaccharomyces pombe* is particularly well-suited for the study of cytokinesis. *S. pombe*, like higher eukaryotes, divides by medial fission that involves the formation of a transient F-actin contractile ring (Marks and Hyams, 1985). The ring can be visualized soon after onset of mitosis and it persists until completion of cytokinesis. During the period when the ring is visualized, its diameter decreases with passage of time. This decrease in diameter has been interpreted to reflect a contractile nature of the ring (Alfa and Hyams, 1990). Experiments with reverting protoplasts have also suggested that the *S. pombe* F-actin ring is contractile in nature (Jochova et al., 1991). The contractile ring is visualized earlier than the medial septum, and has been proposed to guide septum positioning (Marks and Hyams, 1985). In cells that are not undergoing cytokinesis and septation, F-actin is visualized in punctate structures at the tips of the cell where active cell wall deposition occurs.

In an initial genetic screen for *S. pombe* mutants defective for cell cycle–progression, mutations at eight loci were

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identified which resulted in failure of cells to complete cytokinesis and cell separation. DNA replication and mitosis were not affected in these mutants (Nurse et al., 1976). These mutants were grouped into two categories. The *cdc7*, *cdc11*, *cdc14*, and *cdc15*, mutants do not accumulate any deposits of septum material and were termed early septation mutants. In contrast, the *cdc3*, *cdc4*, *cdc8*, and *cdc12* mutants form both aberrant septa as well as irregular deposits of septum material, and were thus termed late septation mutants. Subsequent studies have revealed that the late septation mutants appear to be defective in the formation of the F-actin contractile ring, whereas the early septation mutants are defective for contraction of the ring or deposition of septum material (Marks et al., 1987; McCollum, D., and K. Gould, manuscript in preparation). The *cdc3* and *cdc8* genes have been cloned, and the functions and intracellular distributions of their gene products are consistent with roles in the formation or function of the contractile ring. The *cdc3* gene encodes the actin monomer-binding protein, profilin, which is thought to catalyze actin filament formation (Balasubramanian et al., 1994). The *cdc8* gene encodes a novel tropomyosin, an F-actin-binding protein, which appears to be a component of the F-actin ring (Balasubramanian et al., 1992).

Here we have characterized another cytokinesis gene, *cdc4*. We report that the *cdc4* gene product, Cdc4p, is an EF-hand protein. Fluorescence microscopy indicated that Cdc4p is present in the contractile ring at cytokinesis. Biochemical studies have demonstrated that Cdc4p complexes with a 200-kD protein. Like myosin heavy chains, the 200-kD protein can be cross-linked to UTP. These observations and analysis of the predicted sequence of Cdc4p lead us to suggest that Cdc4p belongs to a novel class of myosin light chains essential for cytokinesis.

Materials and Methods

Strains, Media, and Genetic Methods

The genotypes of *S. pombe* strains and their origins are summarized in Table I. Growth media and genetic methods were as described by Moreno et al. (1991). Diploid *cdc4::ura4/cdc4^{ts}* strains were constructed by crossing diploid *cdc4:ura4/cdc4⁺* to haploid *cdc4^{ts}* strains.

Yeast Transformation and Cloning by Complementation

DNA-mediated transformations were performed by the spheroplast

method (Moreno et al., 1991) or by electroporation (Prentice, 1992). An *S. pombe* genomic library constructed in pWH5 (Wright et al., 1986) was a gift of Dr. P. G. Young (Queen's University at Kingston, Kingston, Ontario, Canada). Plasmid WH5 carries the *Saccharomyces cerevisiae* *LEU2* gene which complements the *S. pombe* *leu1-32* mutation. *cdc4-8 leu1-32* cells were transformed with library DNA, and transformants were selected on Edinburgh minimal medium (EMM) + 1.2-M sorbitol plates lacking leucine at 25°C. Transformants were subsequently tested for their ability to form colonies at the restrictive temperature (36°C) on EMM plates lacking leucine. 2 of ~10,000 transformants formed colonies at 36°C. One colony appeared to have arisen from integration of the rescuing sequence and was not analyzed further. Plasmid DNA from the other colony (pMB401) was amplified in *Escherichia coli*. Plasmid MB401 carried 10.5 kb of *S. pombe* DNA. The region of the 10.5-kb insert responsible for complementation of *cdc4-8* was identified by constructing subclones in pSK(-) and testing these for complementation of *cdc4-8 leu1-32* cells by cotransformation with pWH5. *cdc4⁺* cDNA clones were isolated by complementation from an *S. pombe* cDNA expression library (gift of Drs. B. Edgar and C. Norbury, ICRF Cell Cycle Group, Oxford, United Kingdom). The library was constructed in a modified version of pREP3 (Forsburg, 1993; Maundrell, 1993). Expression was under the control of the *nmt1* promoter. Library DNA was introduced into *cdc4-8 leu1-32* cells by electroporation and transformants were selected on EMM plates lacking leucine and supplemented with thiamine to 5 μM to repress transcription from the *nmt1* promoter (Maundrell, 1989). Transformants were replica plated to media with or without thiamine and held at the restrictive temperature to allow colony formation of rescued strains. Colonies appeared on both media. All clones were subsequently confirmed to carry cDNAs representing the *cdc4* locus.

Molecular Biology Methods

Standard techniques for DNA manipulation and bacterial transformations were used (Sambrook et al., 1989). The nucleotide sequence of the *cdc4⁺* gene was determined on both strands using an automated DNA sequencer (model 370A; Applied Biosystems, Inc., Foster City, CA). Database searching was performed using the FASTA program (University of Wisconsin Genetics Computer Group, Madison, WI) (Pearson and Lipman, 1988) using the Cdc4p-predicted amino acid sequence as the query. Sequence comparisons using a clustering analysis were done using the program, PILEUP (University of Wisconsin Genetics Computer Group) (Sneath and Sokal, 1973). To clone mutant alleles of *cdc4*, chromosomal DNA from the mutant strains was isolated as described (Moreno et al., 1991) and the *cdc4* loci amplified by PCR using oligonucleotides 4MUT5' (5' TAGATCCATTAGAAATAGTCAAAC 3') and cdc4END (5' GAAACTATGTAGATTTAATTA 3'). Amplified DNA was purified by gel electrophoresis and the nucleotide sequence was determined.

Construction of a *cdc4::ura4* Strain

Plasmid MB419(+) was created by replacing the 341-bp NcoI-BglII fragment of pMB418, and encoding 84 internal amino acids of Cdc4p, with a 1.8-kb fragment containing the *ura4⁺* gene. The 3.7-kb XbaI-XhoI fragment of pMB419(+) carrying the *cdc4::ura4* allele was used to transform the diploid strain of genotype *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 h⁺/h⁻* to uracil prototrophy. Transformants were replica plated five times at 1-d intervals to medium containing uracil to allow loss

Table I. *S. pombe* Strain List

Strain	Genotype	Source
KGY28	972 <i>h⁻</i>	P. Nurse
KGY180	<i>cdc4-8 h⁻</i>	P. Nurse
KGY232	<i>cdc4-31 h⁻</i>	P. Nurse
YDM27	<i>cdc4-A1 ura4-D18 leu1-32 ade6⁻h⁻</i>	This study
MBY20	<i>cdc4-8 leu1-32 h⁻</i>	This study
YDM62	<i>cdc4-8 lys1-131 mei1-102</i>	This study
MBY34	<i>ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 h⁺/h⁻</i>	This study
MBY40	<i>cdc4::ura4/cdc4⁺</i> MBY34	This study
MBY68	<i>cdc4::ura4/cdc4-31</i> MBY34	This study
MBY69	<i>cdc4::ura4/cdc4-8 ade6-210/ade6-216 leu1-32/leu1⁺ ura4-D18/ura4-D18 h⁺/h⁻</i>	This study
YDM43	<i>cdc4-31 ade6-210 h⁻</i>	This study
MBY70	YDM62/YDM43	This study

of any autonomously replicating DNA molecules carrying the *ura4⁺* gene. Transformants were subsequently replica plated to medium lacking uracil, and colonies that had remained uracil prototrophs were treated as putative stable integrants. Putative integrants were allowed to sporulate by shifting to malt extract plates. Tetrads dissected from one stable integrant displayed a 2:2 segregation of spores that produced *ura⁻* colonies and spores that failed to produce colonies. All spores that produced colonies were *ura⁻*. Southern analysis of genomic DNA confirmed that the diploid strain was heterozygous at the *cdc4* locus, and was therefore of the genotype *cdc4::ura4/cdc4⁺*.

Expression in *E. coli*

A glutathione S-transferase (GST)¹-*cdc4* fusion gene was expressed in *E. coli*. Complementary DNA encoding amino acids 11–121 was amplified by the PCR using oligonucleotides H-421 which introduced a BamHI site (5'-CGCGCGGGATCCGCTTTTCTTTATTTGACCGC-3'), and H-422 which introduced a BglII site (5'-CCGCGCGAGATCTGGACCCTT-TAATAACTCGTCCAT-3'). The amplified product was digested with BamHI and BglII and inserted into the BamHI site of the GST fusion protein expression vector pGEX2T (Smith and Johnson, 1988) to produce plasmid pMB420. The GST-Cdc4 fusion protein was soluble in aqueous solution and was purified by affinity chromatography on a glutathione-agarose column.

Antibodies

A rabbit polyclonal antiserum (H22) was raised by subcutaneous injection of 750 µg of the fusion protein emulsified in Freund's incomplete adjuvant. Booster injections (200 µg of the fusion protein) were administered after 4 and 6 wks. Antibodies specific to the GST portion of the fusion were removed by adsorption of the serum to Sepharose 4B-coupled GST protein. The depleted serum was then passed over a column of Sepharose 4B-coupled GST-Cdc4p fusion protein. The column was washed extensively and the bound antibodies were eluted with 0.15 M glycine, pH 2.5, directly into tubes containing 0.1 M Tris, pH 8.0. Immunoblot analyses using whole-cell protein extracts demonstrated that the affinity-purified antibodies recognized a single polypeptide of apparent relative molecular mass of 14,000. The intensity of signal in this band was increased when extracts from cells overproducing Cdc4p were immunoblotted. Thus, the affinity-purified antiserum H-22 specifically recognizes *S. pombe* Cdc4p.

Fluorescence Microscopy

All fluorescence microscopy was performed using an axioscope (Carl Zeiss, Inc., Thornwood, NY) and the appropriate set of filters. Cell-wall material, DNA, and F-actin were visualized using calcofluor, 4',6-diamidino-2-phenylindole (DAPI), and rhodamine-conjugated phalloidin, respectively. Double staining with calcofluor and DAPI was carried out as described (Alfa et al., 1992). Staining with rhodamine-conjugated phalloidin was performed as described (Marks and Hyams, 1985). For immunostaining, cells were fixed with a mixture of formaldehyde and glutaraldehyde (Moreno et al., 1991), stained with either serum or affinity-purified primary antibodies followed by a Texas red-conjugated goat anti-rabbit IgG secondary antibody. Tri-X Pan 400 ASA film (Eastman Kodak Co., Rochester, NY) and Rapitone PI-4 paper (AGFA Corp., Orangeburg, NY) were used for printing microscopic images.

Immunoprecipitation

Wild-type *S. pombe* cells (972 *h⁻*) were labeled with [³⁵S]methionine for 2.5 h, and extracts were prepared under denaturing conditions as described (Moreno et al., 1991), or under nondenaturing conditions with the single modification that labeled cells were lysed in MIP (25 mM Hepes, pH 7.2, 250 mM NaCl, 100 mM Na₂P₂O₇, 1% NP-40, 100 mM NaF, 10 mM EGTA, 5 mM EDTA, 4 µg/ml leupeptin, 1 mM PMSF, and 2 mM benzamide). Similar conditions have been shown previously to solubilize myosin without disruption of myosin heavy and light chain interactions (Yamakita et al., 1994). We later found that the nondenaturing lysis buffer, NP-40 buffer (6 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.2, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na₃VO₄, 4 µg/ml leupeptin,

1 mM PMSF, and 2 mM benzamide), gave similar results and was used for subsequent experiments. Cdc4 protein was immunoprecipitated in MIP using 6 µl of H22 serum followed by protein A beads. Immune complexes were washed six times in MIP and then analyzed by SDS-PAGE followed by fluorography.

Photoaffinity Labeling

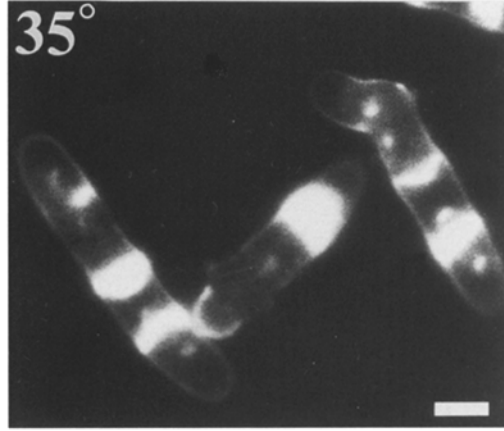
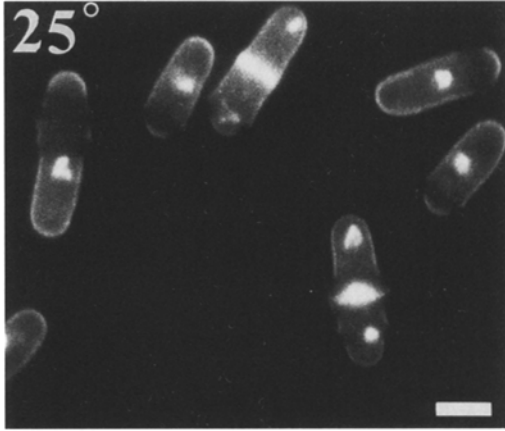
Wild-type *S. pombe* cells (972 *h⁻*) were lysed in NP-40 buffer, and Cdc4 protein immunoprecipitated with H22 serum or with preimmune serum. Immune complexes were washed twice in NP-40 buffer, and then four times in a previously described buffer used for photoaffinity labeling (Gillespie et al., 1993) termed PAL (20 mM KCl, 100 µM CaCl₂, 2.5 mM β-mercaptoethanol, 25 mM Hepes, pH 7.5, 2.5 mM MgCl₂, 1 mM NaVO₄, 1 mM PMSF, 4 µg/ml leupeptin). Immune complexes were suspended in 500 µl of PAL buffer that included 0.2 µM [³²P]UTP (~24 TBq/mmol; ICN Biomedicals Inc., Irvine, CA). Cold ATP was added to 20 µM in competition experiments. Samples were irradiated from above in 24-well tissue culture plates without lids, for 30 min at 4°C with a UV source (UV Stratalinker; Stratagene, Inc., La Jolla, CA). After irradiation, samples were washed three times in PAL buffer, two times in NP-40 buffer, and three times in PAL buffer, then analyzed by SDS-PAGE, and exposed to phosphor storage plates (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

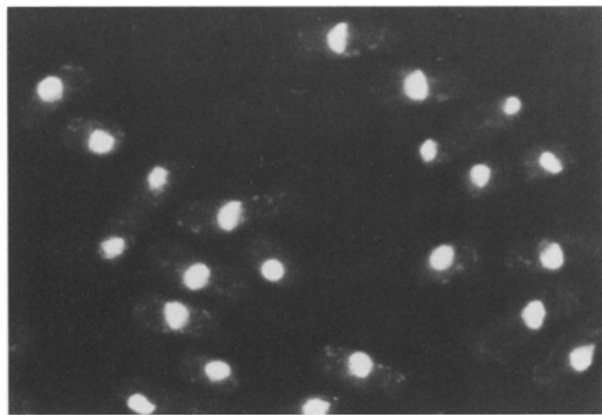
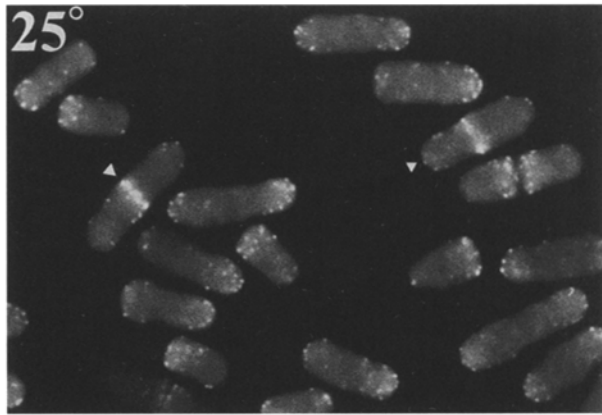
Phenotype of *cdc4* Mutants

Two temperature-sensitive lethal mutant alleles of *cdc4*, *cdc4-8*, and *cdc4-31* were identified in the original screen for cell division control mutants (Nurse et al., 1976). An additional temperature-sensitive lethal allele, *cdc4-A1*, was isolated subsequently (McCollum, D., S. Sazer, and K. Gould, unpublished data). At the restrictive temperature, each of these alleles arrests the cell cycle at cytokinesis, apparently without blocking the nuclear events of the cycle (Nurse et al., 1976). Cells elongate and eventually become dumbbell shaped, accumulating more than two nuclei, and as many as three ill-formed septa without completing cytokinesis (Fig. 1 A). To probe the F-actin architecture in *cdc4-8* cells grown at the permissive temperature or arrested at the restrictive temperature, cells were fixed and stained with rhodamine-conjugated phalloidin and examined by fluorescence microscopy. F-actin staining of *cdc4-8* cells grown at 25°C resembled that reported previously for wild-type cells (Marks & Hyams, 1985). In uninucleate cells, F-actin staining was punctate, appearing in dots and patches near the growing end(s) of cells (Fig. 1 B). In binucleate cells, the staining near the cell poles was decreased, and F-actin was visualized at the medial plane in the contractile ring (Fig. 1 B). Arrested *cdc4-8* cells displayed a markedly different pattern of F-actin staining. In binucleate cells, staining was concentrated between the nuclei but normal contractile rings were not seen. Instead, F-actin staining appeared in cable-like structures (Fig. 1 C). By changing the focal plane it was apparent in some cells that these cables followed the circumference of the cell, suggesting that they were improperly formed contractile rings. In cells with four nuclei, similar improperly formed rings were observed between each pair of nuclei (Fig. 1 C). The septa in these cells were thicker and less regular in shape than in wild-type cells. In many cells, F-actin cables were seen elsewhere in the cell in random orientations (Fig. 1 C). It is known that the formation of the contractile ring precedes formation of the septa and that the position of the contractile ring may determine the

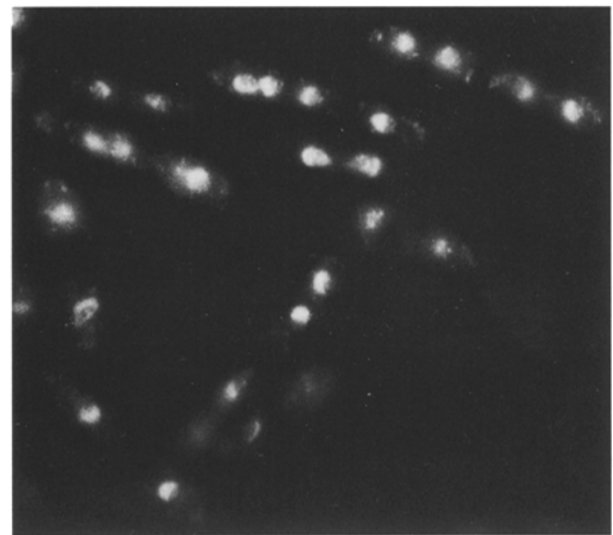
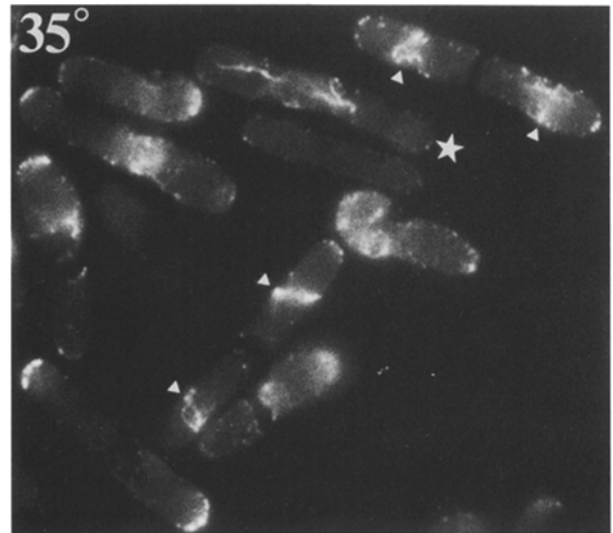
A



B



C



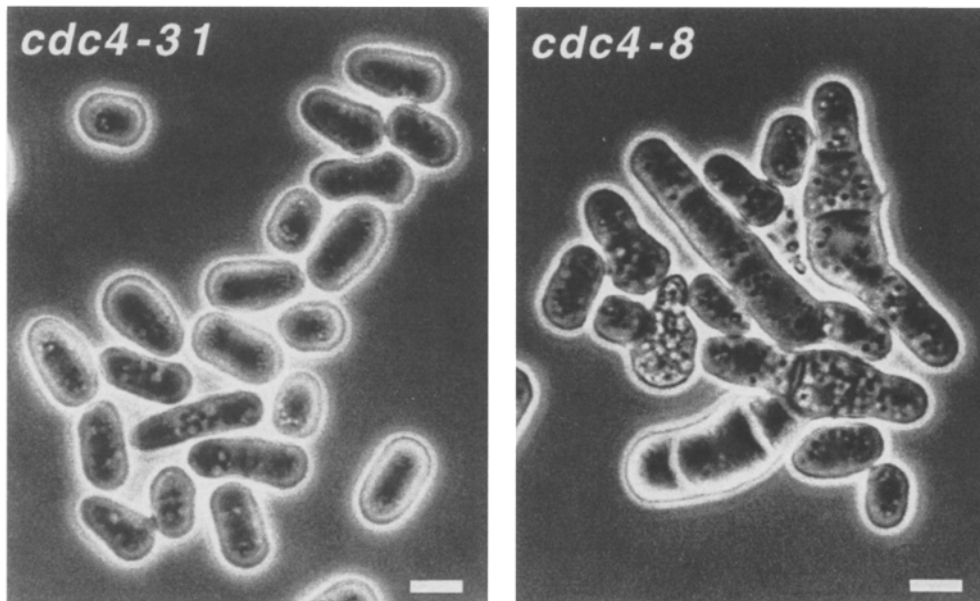


Figure 2. Aberrant morphology of *cdc4-8* cells after nutritional down-shift. *cdc4-8* or *cdc4-31* cells grown in rich medium (yeast extract) were transferred to poor nutritional conditions (malt extract) and allowed to grow overnight at 25°C. Cells were then scraped from these plates, examined and photographed under the phase contrast setting of the microscope. Bar, 5 μ m.

position at which the septum is formed (Marks and Hyams, 1985). A primary defect in contractile ring formation or function in *cdc4* mutants might then account for our observations.

We have also observed a previously undescribed phenotype associated with *cdc4^{ts}* alleles which is most penetrant in the *cdc4-8* mutant. This phenotype was observed at the permissive temperature. After shift to a poorer or richer medium, *cdc4-8* cells became elongated, irregular in diameter, and multiseptated (Fig. 2). 16 h after a shift from yeast extract (rich) medium to malt extract (poor) medium, out of 200 cells counted, 83% of *cdc4-8* cells displayed this phenotype compared with only 15 and 13% for *cdc4-A1* and *cdc4-31*, respectively. Wild-type cells incubated under similar conditions did not display this phenotype. After growth for a few generations under the new conditions, the aberrant *cdc4^{ts}* cells returned to a normal morphology.

Molecular Cloning and Characterization of *cdc4⁺*

An *S. pombe* gene was cloned by functional complementation of the temperature-sensitive lethality of the *cdc4-8* mutant as described in Materials and Methods. The nucleotide sequence of a 2.2-kb StuI-XbaI fragment that complemented *cdc4-8* was determined. (These sequence data are available from GenBank/EMBL/DBJ under accession number L42454.) Examination of the sequence identified a reading frame present in five predicted exons capable of encoding a 141-amino acid (aa) polypeptide. Complementary DNA clones were also isolated by functional complementation of the *cdc4-8* mutant and their nucleotide sequences were determined. This analysis confirmed

the existence and position of the four introns. Disruption of the genomic sequence by deletion of 84 codons and insertion of the *ura4⁺* gene destroyed the ability of this gene to complement *cdc4-8*. Thus, the predicted coding region is responsible for complementation of *cdc4-8*.

Plasmid MB401 DNA, carrying a copy of the cloned, complementing gene and the marker gene *LEU2*, was integrated into the genome of a *cdc4-8* strain by homologous recombination. Crosses between this integrant and wild-type cells failed to segregate *cdc4-8* progeny among 300 spores, indicating that the cloned complementing gene and the *cdc4* locus are identical or tightly linked. A probe derived from the cloned, complementing gene hybridized to probe 8b5, cosmid 30H9c, and P1 clone 8A3p in two ordered genomic *S. pombe* libraries (Maier et al., 1992; Hoheisel et al., 1993). These physical localization data are also consistent with identity or tight linkage between the cloned complementing gene and the *cdc4* locus (Lennon and Lehrach, 1992). Diploids heterozygous for either the *cdc4-8* or *cdc4-31* allele and the disrupted allele of the cloned, complementing gene were constructed. These diploids were temperature sensitive for colony formation at 36°C as would be expected if the cloned gene was *cdc4⁺*, since *cdc4-8* and *cdc4-31* are known to be recessive alleles.

On the basis of the low frequency of recombination between the *cdc4-8* and *cdc4-31* loci, as well as the observation that heterozygous diploid cells were phenotypically wild type, it was proposed that *cdc4-8* and *cdc4-31* were alleles and that they complemented intragenically (Nurse et al., 1976). It remained possible, however, that these two mutants were in very tightly linked genes with related functions. To test this we have constructed a stable het-

Figure 1. F-actin and septum staining in *cdc4-8* cells. *cdc4-8* mutants 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 was maintained at 25°C. After 4 h, cells were stained with rhodamine-conjugated phalloidin for F-actin or with calcofluor for septum material. In both cases, DAPI was included to visualize the chromosomal DNA. (A) Septum/nuclear staining in *cdc4-8* cells at 25°C or at 36°C. (B) F-actin (upper panel) and nuclear staining (lower panel) in *cdc4-8* cells at 25°C. Arrows indicate binucleate cells with actin rings. (C) F-actin (upper panel) and nuclear staining (lower panel) in *cdc4-8* cells at 36°C. Star indicates a cell with random actin cables. Arrows indicate aberrant actin rings. All cells are shown at the same magnification. Bar, 5 μ m.

erozygous diploid (*cdc4-8/cdc4-31 lys1-131/lys1⁺ ade6⁺/ade6-210 meil-102/h⁻*), and confirmed that this strain is capable of colony formation at 36°C. We have constructed the null allele, *cdc4::ura4*, and observed that both *cdc4-8/cdc4::ura4*, and *cdc4-31/cdc4::ura4* diploids are temperature-sensitive lethals. Furthermore, both the *cdc4-8* and *cdc4-31* mutant strains can be rescued by a plasmid expressing the *cdc4* cDNA. These observations are consistent with allelism. Determination of the nucleotide sequence of the *cdc4* locus from *cdc4-8* and *cdc4-31* strains is also consistent with allelism. The *cdc4-31* and *cdc4-8* mutations result in the substitution of glutamic acid for glycine at aa 19, and serine for glycine at aa 107, respectively. Nucleotide sequence of the *cdc4* locus from *cdc4-A1* was also determined, and this mutation changes the glycine at aa 82 to aspartic acid.

The 141-aa Cdc4p sequence was used as the query sequence to search the most recent databases. The databases contain numerous similar sequences, including myosin essential and regulatory light chains and calmodulin. Cdc4p is 38% identical to human skeletal muscle myosin essential light chain (MELC), 26% identical to chicken myosin regulatory light chain (MRLC), and 40% identical to tomato calmodulin (Fig. 3). Unlike calmodulin, which has four functional calcium-binding loops, three of the four potential calcium-binding loops of Cdc4p lack residues known to be necessary for calcium ion coordination. Compared with calmodulin, the second potential calcium-binding loops of many MELCs and MRLCs, respectively, are characterized by a two-aa insertion and a four-aa deletion. Cdc4p resembles MRLC in this regard. Also similar to many MRLCs, the fourth potential calcium-binding loop of Cdc4p begins with a proline. Thus, Cdc4p appears to be a distinct EF-hand protein. Consistent with this suggestion, comparison of Cdc4p with 115 MELC, MRLC, and calmodulin sequences using the program PILEUP in the UWGCG suite of programs (Sneath and Sokal, 1973) produces a dendrogram which places Cdc4p in its own cluster as an outgroup.

cdc4-null Mutants Arrest the Cell Cycle at Cytokinesis

The existence of recessive temperature-sensitive lethal mutations in *cdc4* suggested that this gene is essential, and the terminal phenotypes of these mutants suggested that

Cdc4p is required for completion of cytokinesis. To investigate further the requirement for *cdc4* function, strains bearing the null allele were analyzed. The meiotic products of *cdc4::ura4/cdc4⁺* diploid cells produced haploid colonies, all of which were Ura⁻. Microscopic examination revealed that the presumed Ura⁺ spores had germinated and arrested the cell cycle as single cells after becoming elongated and dumbbell-shaped, suggesting that the predominant function of Cdc4p is in cytokinesis. An independent assessment of the effect of loss of *cdc4* function was made by constructing a haploid strain *cdc4::ura4* rescued by a plasmid-borne copy of *cdc4⁺* (pMB401). After a period of growth without maintaining selection for the plasmid, microscopic examination revealed that some of the cells, presumably those which lost pMB401, had arrested with an elongated, dumbbell-shaped morphology (Fig. 4). Most *cdc4::ura4* cells accumulated multiple nuclei and displayed punctate F-actin staining both in the peripheral and medial regions of the cell. However, whereas *cdc4^{ts}* mutants accumulated improperly formed contractile rings and randomly oriented cable-like structures, these were not observed in either binucleate (Fig. 4 A) or tetranucleate (Fig. 4 B) *cdc4*-null mutant cells. Septum material accumulated in the medial region of the null mutants, but properly formed septa were not observed (not shown). These observations establish that Cdc4p is essential for viability and that it is required, apparently specifically, for F-actin contractile ring function.

Cdc4p Localizes to the Contractile Ring

Antibodies were generated against a GST-Cdc4 fusion protein for determination of the distribution of Cdc4p by indirect immunofluorescence microscopy. Wild-type cells were stained with either preimmune or immune serum. Preimmune serum produced only a low level, uniform, diffuse staining of the cell, similar to that produced by the secondary antibody alone. In contrast, immune serum produced distinct staining. Some interphase cells displayed low intensity punctate staining as in cells 1 and 8 (Fig. 5). However, most were similar in appearance to cells stained with preimmune serum. Soon after initiation of mitosis, as judged by DAPI staining, a band of immunoreactive material became visible in the medial region of the cell (see cells 2 and 3, Fig. 5). Upon viewing in several focal planes, the structure appeared as a ring. These ring structures

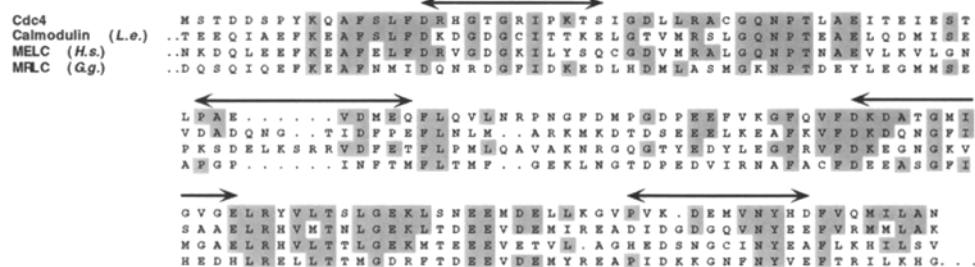


Figure 3. Alignment between predicted amino acid sequence of Cdc4p and three other EF-hand proteins (calmodulin from *Lycopersicon esculantum*, MELC from *Homo sapiens*, and MRLC from *Gallus gallus*). Bidirectional arrows represent amino acid residues within calcium-binding loops of calmodulins. Amino acid residues identical between Cdc4p and at least one other EF-hand protein have been boxed. Gaps and other features of the sequence have been described in the text.

were observed only in cells with two nuclei or condensed chromatin. Later in mitosis, after separation of the chromatin, the medial staining decreased progressively in diameter (cells 4–6, Fig. 5). Finally, only a single dot of immunoreactive material was visible in the medial region of the cell (cell 7, Fig. 5). After completion of cytokinesis, Cdc4p staining again became diffuse. Cdc4p ring staining was observed in 85 of 500 cells (17%). This number correlates well with the percentage of stainable F-actin rings in an asynchronous culture (Jochova et al., 1991). The same staining pattern was observed using affinity-purified anti-Cdc4p antibodies although the staining was not as intense, possibly as a result of loss of the highest affinity antibodies during affinity purification. Thus Cdc4p appears to be a component of the contractile ring. Interestingly, Cdc4p was also found to localize to the aberrant rings found in *cdc4-8* mutants at the restrictive temperature (data not shown).

Cdc4p Complexes with a 200-kD Protein In Vivo

Since Cdc4p has sequence similarity to myosin light chains, and is localized to the region of the contractile ring, we wished to determine if it was complexed with a protein of similar size to myosin heavy chain in vivo. Extracts of cells were prepared under nondenaturing conditions which had been shown previously to solubilize myosin without disrupting the association between the heavy and light chains. To determine if Cdc4p was solubilized completely under these conditions, wild-type cells labeled with [³⁵S]methionine were lysed, and an insoluble pellet fraction and a soluble supernatant fraction were prepared by centrifugation. The pellet and soluble fractions were denatured and Cdc4p was immunoprecipitated and analyzed by SDS-PAGE. Cdc4p was found almost exclusively in the soluble supernatant fraction (data not shown), indicating that solubilization had been complete. Anti-Cdc4p antibodies were then used to immunoprecipitate material from this soluble fraction under nondenaturing conditions and from a total extract made under denaturing conditions. Parallel immunoprecipitations were carried out with preimmune serum. As expected, Cdc4p was specifically immunoprecipitated from the denatured extract (Fig. 6 A). However, a group of three major polypeptides of ~200 kD, and two minor polypeptides of 110 and 50 kD coimmunoprecipitated with Cdc4p under nondenaturing conditions (Fig. 6 A). The recovery of the lower two polypeptides was variable, and they may be degradation products of the upper polypeptide. The 200-kD protein might be a myosin heavy

chain. However, attempts to identify it as myosin using immunological methods have been unsuccessful.

In shorter exposures, it was clear that Cdc4p immunoprecipitated from the denatured extract consisted of two forms: a major form of lower apparent molecular weight, and a minor form of slightly higher apparent molecular weight. Immunoblotting of immunoprecipitated, unlabeled protein from denatured extracts produced the same doublet. Quantification of the relative band intensities revealed that the minor form constituted 13% of the Cdc4 protein immunoprecipitated under these conditions. Surprisingly, the minor form of the protein was not immunoprecipitated from nondenatured extracts. Although it is possible that the minor form may be inaccessible to the antibodies under nondenaturing conditions, mixing experiments suggested that it was unstable under these conditions (data not shown).

A number of myosin heavy chains have been shown to be capable of being photo-cross-linked to UTP (Maruta and Korn, 1981; Gillespie et al., 1993). For this reason, we wished to determine if the 200-kD protein that complexed with Cdc4p could be photo-cross-linked to UTP. Lysates were prepared from wild-type cells under nondenaturing conditions, and Cdc4p was immunoprecipitated using α -Cdc4p antibodies, with preimmune serum used as a control. Immune complexes were incubated with [α -³²P]UTP and irradiated with UV light. We found that a 200-kD protein was labeled specifically after irradiation of immune complexes prepared with immune serum (Fig. 6 B). Labeling was not observed when the experiment was performed with preimmune serum (Fig. 6 B). The 200-kD protein was not labeled either in the absence of UV irradiation, or when 100-fold excess of unlabeled ATP was present (Fig. 6 B).

Discussion

Our analysis of temperature-sensitive *cdc4* mutants showed that they arrested with multiple nuclei separated by aberrant contractile rings. The rings found in arrested *cdc4^{ts}* cells were irregular in shape and improperly oriented. These initial observations suggested that Cdc4p function is required for the proper organization and placement of the ring, but that it is not required for ring assembly per se. Analysis of the *cdc4*-null allele showed that Cdc4p is essential and confirmed that it is required for ring function. The null allele-terminal phenotype differed from that of the conditional mutants, in that improperly formed rings or cables were not observed. Although the possibility

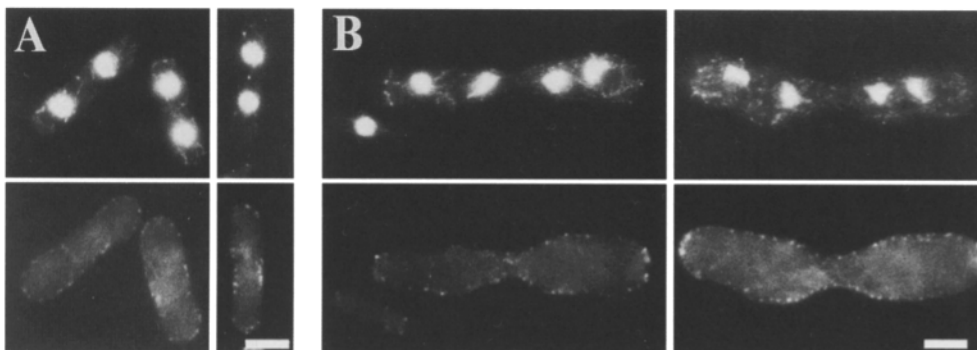


Figure 4. The phenotype of *cdc4* null cells. Null mutant cells generated by plasmid loss, as described in results, were stained for F-actin using rhodamine-conjugated phalloidin (bottom panel) and DNA using DAPI (top panel). Both binucleate (A) and tetranucleate (B) cells are shown. All cells are shown at the same magnification. Bar, 5 μ m.

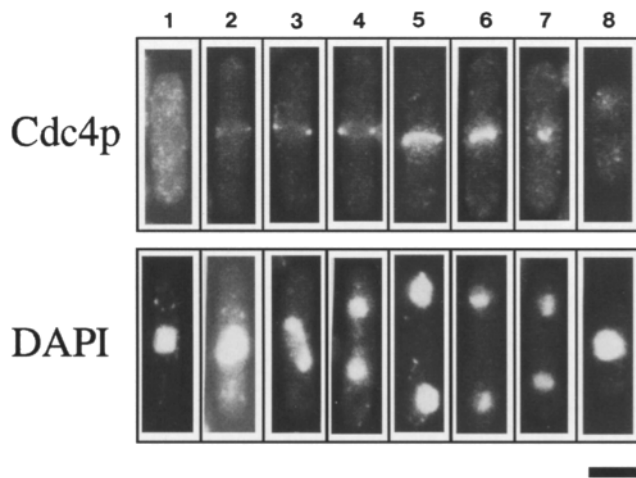


Figure 5. Localization of Cdc4p during the cell cycle. Wild-type cells were fixed with formaldehyde/glutaraldehyde and stained for Cdc4 protein using H22 antiserum followed by Texas red-conjugated secondary antibodies. DNA was stained using DAPI. Cells at differing stages of the cell cycle are shown. Cells 1 and 2 are uninucleate interphase cells. Cells 2–7 are in progressive stages of mitosis/cytokinesis, beginning in early mitosis (cell 2) and progressing to late mitosis and cytokinesis (cell 7). All cells are shown at the same magnification. Bar, 5 μ m.

remains that the ring structure is unstable under the conditions used for fixing and staining cells if Cdc4p is not present, it seems likely that Cdc4p function is essential for actin ring formation. In either case, the presence of improperly formed rings and F-actin cables in blocked *cdc4-8* and *cdc4-31* cells suggests that these alleles are hypomorphic.

In *S. pombe*, mitosis and cytokinesis are coupled such that acceleration or deceleration of entry into mitosis, which occurs upon nutritional down-shift or up-shift, results in acceleration or deceleration of entry into cytokinesis (Nurse, 1975). The *cdc4-8* allele, and to a lesser extent the *cdc4-31* and *cdc4-A1* alleles, showed a non-wild-type response to nutritional up-shift or down-shift at the permissive temperature for growth. These observations suggest that Cdc4p activity may be involved in the coupling mechanism and that Cdc4-8p, and to a lesser extent Cdc4-31p and Cdc4-A1p, appear to be defective for this activity.

Immunofluorescence data suggested that Cdc4p is a component of the F-actin contractile ring. In wild-type cells, Cdc4p staining was seen in the form of a band or a ring only at the medial plane of cells undergoing mitosis and cytokinesis. The diameter of the visualized ring was smaller in cells that were fixed at progressively later stages of cytokinesis and septation. Both of these features are shared between the Cdc4p-ring and the F-actin ring (Marks et al., 1985). However, unlike F-actin, punctate Cdc4p staining was not detected at regions of cell-wall deposition in interphase cells. The immunolocalization results taken together with the specific arrest of *cdc4* null mutants at cytokinesis suggests that Cdc4p is required for F-actin contractile ring function and not for other aspects of F-actin function. Genetic evidence also supports a role for Cdc4p in actin ring function. Strong negative interactions have been identified between *cdc4-8* and *cdc8-110* (McCollum, D., and K. Gould, manuscript in preparation). The *cdc8*

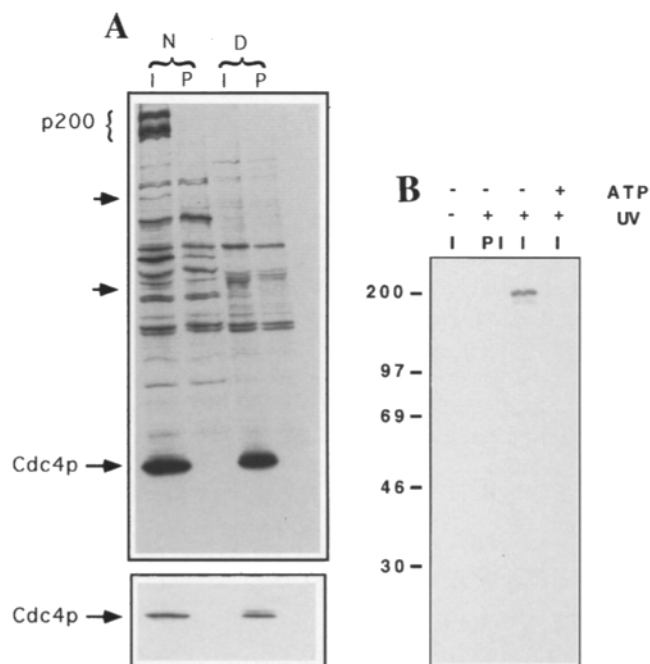


Figure 6. (A) Cdc4p complexes with a 200-kD protein. Wild-type cells were labeled with 35 S and Cdc4p was immunoprecipitated under nondenaturing (N) or denaturing (D) conditions, using either immune serum (I) or preimmune serum (PI) as a control. The 200-kD protein (shown in brackets) as well as two less abundant proteins (arrows) found specifically in Cdc4p immune complexes are shown. (Lower panel) A shorter exposure of the above gel showing that two forms of Cdc4p were seen under denaturing conditions. (B) Photoaffinity labeling of the 200-kD protein complexed with Cdc4p. The 200-kD protein complexed with Cdc4p was immunoprecipitated from wild-type protein lysates using preimmune (PI), or immune (I) Cdc4p serum. Immunoprecipitates were incubated in the presence of [α - 32 P]UTP, with (+) or without (–) a 100-fold excess of cold ATP. Samples were then treated either with (+) or without (–) UV irradiation.

gene encodes a novel tropomyosin, an F-actin-binding protein, which localizes to the contractile ring, and is essential for its function (Balasubramanian et al., 1992). These observations are consistent, then, with the presence of both Cdc8p and Cdc4p in the contractile ring.

Inspection of the predicted sequence of Cdc4p indicated that it is a member of the EF-hand family of proteins which includes the myosin light chains, calmodulin, and troponin C. The Cdc4p sequence has some of the features that are unique to MELC and some that are unique to MRLC. Since three out of four calcium-binding loops lack residues essential for calcium binding, Cdc4p cannot be a calmodulin or a troponin C. A clustering analysis based on comparison of the Cdc4p sequence with 33 MELC, 25 MRLC, 14 troponin C, 46 calmodulin, and 8 other miscellaneous EF-hand protein sequences suggests that Cdc4p diverged from a branch that encompasses troponin C, calmodulin, and MELC. No other sequences in the databases clustered with that of Cdc4p. This suggests the possibility that Cdc4p is a prototype of a novel class of EF-hand proteins.

Prototypic MELC and MRLC, which have been shown to function in cytokinesis, have not been identified in

yeasts. *Dictyostelium* mutants lacking either the MELC or the MRLC are defective for cytokinesis (Pollenz et al., 1992; Chen et al., 1994), and mutations in the *Drosophila* spaghetti-squash gene, which encodes a MRLC, also lead to defective cytokinesis (Karess et al., 1991). As a first step to address if Cdc4p is indeed a myosin light chain, we sought to identify proteins that physically associate with Cdc4p. Antibodies to Cdc4p specifically coimmunoprecipitated a polypeptide with a size similar to that of myosin heavy chains (200 kD). The 200-kD protein can be photo-cross-linked to UTP which is a property common to myosin heavy chains. However, antibodies directed against a variety of known myosins did not display reactivity to the 200-kD protein. Antibodies to *S. pombe* myosin are not available. Antibodies raised against type II myosins from *S. cerevisiae*, *D. discoideum*, and *Caenorhabditis elegans*, and type V myosin from *S. cerevisiae* were not cross-reactive. Furthermore, a pool of monoclonals raised against *Acanthamoeba* type II myosin, which recognize the head domain from many myosins, are nonreactive against the 200-kD protein. Thus, we have not been able to confirm that the 200-kD protein is myosin. Efforts are currently under way to establish the molecular identity of this 200-kD protein.

A large number of studies have implicated the type II myosin as the form that is involved in cytokinesis (Satterwhite and Pollard, 1992). Type II myosin is composed of two regulatory light chains, and two essential light chains associated with two myosin heavy chains (Harrington and Rodgers, 1984). If the 200-kD protein is a type II myosin, then it is surprising that we do not observe a second light chain in the immunoprecipitated (Cdc4p/p200) complex. This could be explained if p200 is not a type II myosin, or if the second light chain comigrates with Cdc4p on our gel system. Further biochemical analysis will be required to distinguish between these possibilities.

The assembly and activity of the contractile ring is regulated both temporally and spatially in a cell cycle-dependent manner. Thus, Cdc4 localization must also be regulated in a cell cycle-dependent manner. Western blotting of extracts from cells synchronized by centrifugal elutriation showed that the levels of Cdc4p do not vary appreciably throughout the cell cycle (data not shown), indicating that Cdc4p localization must be regulated posttranslationally. In denatured extracts from asynchronous wild-type cells, 13% of the Cdc4 protein was found in a form of slightly higher apparent molecular weight. This form was found to be unstable under non-denaturing buffer conditions, suggesting that it arose from a posttranslational modification. Interestingly, the percentage of Cdc4p found in the slower migrating form (13%) is similar to the percentage of cells in an asynchronous culture that displayed Cdc4p ring staining (17%). This raises the intriguing possibility that the slower migrating form of Cdc4p may represent the form of the protein present in the contractile ring. Although at present we do not know the nature of the modification in the slower migrating form of the Cdc4 protein, it is tempting to speculate that it is due to phosphorylation, since preliminary studies have shown that Cdc4p is a phosphoprotein (data not shown). In higher eukaryotes, phosphorylation at the amino terminus of the myosin regulatory light chain by the Cdc2 kinase is thought to be one

mechanism by which cytokinesis is regulated (Satterwhite et al., 1992; Yamakita et al., 1994). This result is interesting since sequence analysis identified a consensus Cdc2 kinase phosphorylation site (Nigg, 1991) near the amino terminus of the Cdc4 protein. Thus, it will be interesting in future studies to determine if phosphorylation of Cdc4p by Cdc2p or other kinases is important for regulation of Cdc4p activity, and in turn, cytokinesis.

We have identified the amino acids mutated in the three available, conditionally lethal alleles of *cdc4*. Each of these mutations alters glycine residues conserved among EF-hand proteins (see Results). The observed intragenic complementation between *cdc4-8* and *cdc4-31* might be explained if Cdc4p functions as a dimer, and if a dimer composed of Cdc4p and Cdc4-31p is functional. However, there is no evidence that conventional myosin light chains function as homodimers. Alternatively, it is possible that different domains of Cdc4p perform distinct functions related to contractile ring assembly/stability. Thus, the complementation of the *cdc4-8* and *cdc4-31* mutants may reflect the fact that each allele affects a distinct functional domain. Intragenic complementation has been observed between phenotypically distinct alleles of the *S. cerevisiae* calmodulin, Cmd1p, and has been proposed to reflect the multifunctionality of calmodulin (Ohya and Botstein, 1994). Recently, Cmd1p was shown to be the light chain of a type V myosin, Myo2p in *S. cerevisiae* (Brockerhoff et al., 1994). A strong negative interaction was also identified between the *myo2-66* and *cmd1-8* mutations. Interestingly, *cmd1-8* allele is mutated in the glycine residue corresponding to that mutated in the *cdc4-8* allele. In light of this, it should be interesting to determine if interaction between Cdc4-8p and p200 is disrupted in *cdc4-8* cells.

Mutants defining four *S. pombe* genes (*cdc3*, *cdc4*, *cdc8*, and *cdc12*), termed the late septation mutants, were implicated in cytokinesis (Nurse et al., 1976). Molecular analysis of *cdc3* and *cdc8* identified them as encoding two proteins essential for contractile ring function: the actin-binding proteins, profilin (Balasubramanian et al., 1994), and tropomyosin (Balasubramanian et al., 1992). We have characterized a third late septation gene, *cdc4*, which encodes a novel EF-hand protein, possibly a myosin light chain, that is also an essential component of the contractile ring. At the restrictive temperature, temperature-sensitive alleles of each of these are defective for the formation or the stability of the F-actin contractile ring (McCollum, D., and K. Gould, manuscript in preparation). The identification, in a number of systems, of at least 15 proteins associated with the actin ring (Satterwhite and Pollard, 1992) suggests that the list of genes encoding contractile ring proteins in *S. pombe* is incomplete. Further genetic analysis of the *cdc3*, *cdc4*, *cdc8*, and *cdc12* genes should lead to the identification of other *S. pombe* contractile ring proteins.

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