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Cdc4p, a Contractile Ring Protein Essential for Cytokinesis in *Schizosaccharomyces pombe*, Interacts with a Phosphatidylinositol 4-Kinase*

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Michel Desautels‡§¶, Jason P. Den Haese§‖, Carolyn M. Slupsky‡‡, Lawrence P. McIntosh**, and Sean M. Hemmingsen§¶‖**

*From the Departments of ‡Physiology and ‖Microbiology and Immunology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E5, the §Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, S7N 0W9, and the **Department of Biochemistry and Molecular Biology, Department of Chemistry, and the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada*

The proposed function of Cdc4p, an essential contractile ring protein in *Schizosaccharomyces pombe*, is that of a myosin essential light chain. However, five conditionally lethal *cdc4* alleles exhibit complementation in diploids. Such interallelic complementation is not readily explained if the *sole* function of Cdc4p is that of a myosin essential light chain. Complementation of *cdc4* alleles could occur only if different mutant forms can assemble into an active oligomeric complex or if Cdc4p has more than one essential function. To search for other proteins that may interact with Cdc4p, we performed a two-hybrid screen and identified two such candidates: one similar to *Saccharomyces cerevisiae* Vps27p and the other a putative phosphatidylinositol (PI) 4-kinase. Binding of Cdc4p to the latter and to myosin heavy chain (Myo2p) was confirmed by immunosorbent assays. Deletion studies demonstrated interaction between the Cdc4p C-terminal domain and the PI 4-kinase C-terminal domain. Furthermore, interaction was abolished by the Cdc4p C-terminal domain point mutation, Gly^{107} to Ser. This allele also causes failure of cytokinesis. Ectopic expression of the PI 4-kinase C-terminal domain caused cytokinesis defects that were most extreme in cells carrying the G107S allele. We suggest that Cdc4p plays multiple roles in cytokinesis and that interaction with a PI 4-kinase may be important for contractile ring assembly and/or function.

Cytokinesis, the separation of one cell into two following mitosis, is a complex process requiring spatial and temporal coordination of multiple cell cycle events (1–3). One hallmark of cytokinesis is the assembly of a contractile ring made of actin, myosin, and many other proteins (2–4). The contractile ring is a transient structure that in *Schizosaccharomyces pombe* assembles in the early stages of mitosis and disappears with cell division (4–6). The period between onset of ring assembly and its disappearance is 30–70 min, depending upon growth conditions, or about one quarter of the cell cycle (6). The process is conserved in animal cells with a similar actin-myosin contractile ring acting in cytokinesis (2, 7). Appropriate placement of the ring at the medial plane of the cell is the first critical feature of cytokinesis. It follows initiation of mitosis and requires the contribution of many gene products such as Mid1, Pos1, Pos2, and Pos3 (3, 4). Subsequently, assembly of the ring occurs with recruitment of several cytoskeletal and regulatory proteins (2–4). However, little is known about the contribution of these individual components in the assembly and function of the ring.

One contractile ring protein essential for cytokinesis is Cdc4p. The *cdc4* locus was first identified by Nurse *et al*. (8) in a screen for cell division control mutants. Cells with disruption of the *cdc4* gene or with conditionally lethal point mutations such as G19E or G107S become elongated, accumulate two or more nuclei and ill formed septa, and fail to divide (8, 9). Spectroscopic measurements reveal that these mutations cause only small conformational perturbations, suggesting that the altered phenotypes arise directly from a disruption of the function of Cdc4p rather than indirectly through a destabilization of its structure.[1] Recent studies have shown that a function of Cdc4p is that of a myosin light chain. For example, sequence analysis suggested that Cdc4p was likely an EF-hand protein with similarity to calmodulin and to myosin essential and regulatory light chains (9). We determined recently by NMR spectroscopy that the tertiary structure of Cdc4p is indeed that of a dumbbell-shaped EF-hand protein, composed of two structurally independent domains joined by a flexible linker region.[1] Its precise structure is sufficiently distinct from either the myosin essential light chain (ELC)[2] or regulatory light chain (RLC) of the muscle myosin-ELC-RLC complex (10) that it is not possible on this basis alone to identify Cdc4p as either an RLC or an ELC. However, genetic evidence suggests that Cdc4p binds to myosin heavy chain (Myo2p) at the first IQ

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¶ To whom correspondence may be addressed. Tel.: 306-966-6538; Fax: 306-966-6532; E-mail: desautel@duke.usask.ca.

‡‡ Present address: Protein Engineering Network Centres of Excellence, 713 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, T6G 2S2, Canada.

§§ To whom correspondence may be addressed. Tel.: 306-975-5242; Fax: 306-975-4839; E-mail: hemmings@cbrpbi.pbi.nrc.ca.

[1] Slupsky, C. M., Desautels, M., Huebert, T., Zhao, R., Hemmingsen, S. M., and McIntosh, L. P. (2001) *J. Biol. Chem.* **276,** 5944–5952

[2] The abbreviations used are: ELC, essential light chain; PCR, polymerase chain reaction; PI, phosphatidylinositol; RLC, regulatory light chain; bp, base pair(s); 3AT, 3-amino-1′,2′,4′triazole; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

motif, an established binding site for ELC in conventional myosins (11). Recently, a gene that presumably also encodes a small EF-hand protein with sequence similarity to Cdc4p was annotated as a putative RLC. This was based on sequence similarity to *Drosophila melanogaster* RLC (Sanger Center *S. pombe* sequencing group, accession number CAB54151). This protein localizes to the contractile ring yet binds to Myo2p at the second IQ motif, an established RLC-binding site.[3] Thus, current genetic and biochemical evidence suggests there are two light chains associated with Myo2p: Cdc4p at the ELC position and a second protein at the RLC position.

However, several observations suggest that Cdc4p may be more than just an essential light chain bound to Myo2p. First, although the abundance of Cdc4p does not vary with the cell cycle, its cellular localization does. In interphase cells, Cdc4p is detected as punctuate immunostaining distributed throughout the cells, whereas prior to cell division it is recruited to the contractile ring (9). How this recruitment is achieved is unknown, but it can be independent of Myo2p, because Cdc4p is still found in improperly formed rings in *myo2*-null mutants (11). It is possible that Cdc4p is recruited to the contractile ring in *myo2*-null cells via interaction with Myp2p, a second myosin heavy chain in *S. pombe* that also localizes to the ring (12, 13). Second, a synthetic lethal genetic interaction has been reported between *cdc4* and *rng2*, a gene encoding an essential contractile ring protein similar to human IQGAP1 (14). In budding yeast, a light chain for a type V myosin has been reported to physically associate with an IQGAP-like protein and to mediate its localization to the contractile ring (15). Similarly a human cytoskeletal ELC binds IQGAP1 (16). Thus, it is very likely that Cdc4p physically interacts with Rng2p. Human IQGAP1 is known to bind actin and calmodulin and to regulate Rho GTPases (14). Thus, it is well positioned to link signaling pathways to actin remodeling. Phenotypic analysis of *cdc4* and *rng2* mutants lead to the suggestion that these gene products may be involved in organizing actin cables into a ring (4). Finally, it was observed by Nurse *et al.* (8) that diploid cells bearing both mutant alleles *cdc4-G19E* and *cdc4-G107S* are viable at a restrictive temperature, whereas diploid cells homozygous for either mutant allele alone are not. Such interallelic complementation can be explained if Cdc4p has more than one essential and independent function, each of which is disrupted selectively by a mutation. Alternatively, Cdc4p could serve as an oligomer with cooperative interaction of mutant proteins allowing formation of an active complex. Although there is no evidence that conventional myosin light chains function as homodimers and although purified Cdc4p is clearly a monomer in solution, a structural model with Cdc4p serving as both an ELC and RLC can be developed.[1]

The objective of this work was to further define *cdc4* functions by identifying other *S. pombe* proteins that interact with Cdc4p. In addition to confirming the original observation of interallelic complementation of the *cdc4-G107S* and *cdc4-G19E* alleles in diploid cells, we found several additional pairs of *cdc4* temperature-sensitive alleles that complement. Then, using a yeast two-hybrid screen, we identified interactions between Cdc4p and two proteins: one similar to *Saccharomyces cerevisiae* Vps27p and the other a putative phosphatidylinositol 4-kinase. The latter interaction was confirmed by immunochemical methods and further investigated by deletion studies. Therefore, in addition to its role as a myosin light chain, Cdc4p may participate in interactions that regulate contractile ring assembly.

[3] M. K. Balasubramanian, personal communication.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic and Molecular Biology Methods—*S. pombe* strains routinely used in this study were wild-type h^+ 975 cells, h^- 972 cells, and strains MD38 (h^+ *ade*6-216 *leu*1−32 *ura*4-D18), MD40 (h^+ *cdc*4-8 *ade*6-210 *leu*1-32 *ura*4-D18), and MD42 (h^+ *cdc*4-31 *ade*6-210 *leu*1-32 *ura*4-D18) previously obtained from P. Nurse. Strains carrying *cdc*4 ts^{lethal} alleles (*cdc*4-8, 31, A1, A2, A11, C2; see Fig. 1) in h90 *mam*2::LEU2 background were generously provided by M. K. Balasubramanian. Growth media (yeast extract, malt extract, and Edinburgh minimal medium with supplements) were as described (17). Overexpression of proteins in *S. pombe* was carried out from the thiamine-repressible *nmt*1 promoter in plasmids pRep1 and pRep2 for leucine and uracil prototrophy, respectively (18). *S. pombe* transformations were by electroporation (19). Standard techniques for DNA manipulation, plasmid constructs, and bacterial transformations were used. DNA was sequenced using a model 370A automated sequencer (PE Applied Biosystems Inc.).

Diploid Strains—Strains carrying cdc4 ts^{lethal} alleles (3) in h90 *mam*2::LEU2 background were crossed with h^- 972. Progeny were mated four or five times with h^+ 975 or h^- 972 to obtain strains that were unambiguously h^- or h^+, and ts^{lethal}. These strains were mated with strain h^+ *ade*6-M210 *ura*4-D18 *leu*1-32 *his*3-D1 (ATCC96116) or strain h^- *ade*6-M216 *ura*4-D18 *leu*1-32 *his*3-D1 (ATCC96117) to obtain temperature-sensitive haploid cells of each mating type and various selection markers. Diploid strains were constructed from the haploid strains as described (17). Identification of the cdc4 allele carried by each haploid strain was reconfirmed by PCR amplification and direct sequence determination of the amplified coding region. A list of all diploid strains bearing homozygous and heterozygous combinations of *cdc4* alleles and selection markers is available upon request.

S. pombe cDNA Library for S. cerevisiae Two-hybrid Screen—The library vector, pBI771 (20), was an incremental derivative of pPC86 (21), which is a DNA-binding domain fusion vector based on the *S. cerevisiae* GAL4 gene. Poly(A)$^+$ RNA extracted from *S. pombe* 972 h^- cells in the logarithmic phase of growth was used as template for synthesis of double-stranded cDNA. A *Not*I site was introduced 3′ to the oligo$(dT)_{17}$ sequence of the primer used for reverse transcriptase. After addition of a *Sal*I linker, the double-stranded cDNA was restricted with *Not*I, size fractionated (0.5–5 kilobase pairs), and ligated with pBI771 DNA that had been digested previously with both restriction endonucleases. *Escherichia coli* DH5α cells were transformed with the ligated library DNA and plated on two 25 × 25-cm plates containing 2YT + ampicillin (100 μg/ml). The complexity of the primary library was estimated at 1.5×10^6 independent clones. After incubation overnight, cells were scraped into liquid medium and further incubated for 3 h at 37 °C. This library (JDH-12) was stored at −80 °C. An aliquot was amplified to obtain ~3 mg of library plasmid DNA.

Two-hybrid Screen—The two-hybrid interaction screen of an *S. pombe* cDNA library and subsequent assays for interaction between two specific gene products were carried out exactly as described by Kohalmi *et al.* (20, 22). Bait vector constructs in pBI880 vector included *cdc4,* mutated alleles of *cdc4*, N-terminal and C-terminal domains of *cdc4,* tropomyosin (*cdc8*), and calmodulin (*cam1*), as *Sal*I-PstI or *Sal*I-*Bam*HI inserts. A *cdc4* cDNA construct in an *E. coli* expression vector pRSET B (Invitrogen Corp., San Diego, CA) was a gift of Dr. Dan McCollum (Vanderbilt University). To produce alleles of *cdc4* in the bait vector, PCR-mediated site-directed mutagenesis was used to introduce single nucleotide substitutions in the coding sequence of *cdc4* in the pBI880 vector. The following substitutions were carried out using the oligonucleotide primers indicated in brackets: *cdc4-A2,* T → C at bp 34 (H886-JN069); *cdc4-31*, G → A at bp 56 (H743-JN069); *cdc4-A11*, T → C at bp 236 (BC293-H890,H889-JN069); *cdc4-A1,* G → A at bp 245 (BC293-H746,H747-JN069); *cdc4-8*, G → A at bp 319 (BC293-H763,H762-JN069) (Fig. 1 indicates the conversion between allele name and the resulting amino acid substitution). BC293 is a *Gal4DB*-specific primer, whereas JN069 is an ADH1 terminator primer, used to PCR amplify the entire insert region of the bait vector pBI880. Constructs of *cdc4* N-terminal and C-terminal domains as *Sal*I-*Bam*HI inserts in the bait vector were obtained by PCR amplification of the coding sequence for either the first 72 amino acids or the last 68 amino acids, using *cdc4* in pBI880 vector as template DNA. The primer pair for amplification of the N-terminal domain of Cdc4p was BC293-H836 with the reverse primer designed to include the last five amino acids of Cdc4p and a *Bam*HI restriction site. The primer pair for amplification of the C-terminal domain of Cdc4p was H833-JN069, with the forward primer designed to include the first three amino acids of Cdc4p and a *Sal*I restriction site. The *cam1* gene was obtained from PCR gene amplifi-

cation of *S. pombe* genomic DNA with primer pair H1042 and H1010, introducing a *Sal*I site at the 5′end and a *Pst*I site immediately 3′ to a termination codon. All oligonucleotide sequences are available upon request. All *cdc4* mutations and domain constructs were verified by sequencing. *S. cerevisiae* strain YPB2 was transformed with bait and library vectors by the lithium acetate method (20, 22). Positive interactions were identified first by selection for *HIS3*, shown by cell growth at 25 °C in synthetic dextrose medium lacking histidine and supplemented with 3-amino-1′,2′,4′triazole (3AT). 3AT-resistant clones were then tested for activation of the second gene marker, *lacZ*, by the X-gal colony filter assay (20, 22).

Ectopic Gene Expression in S. pombe—Expression vectors were constructed for the full *cdc4* coding region, for regions of *cdc4* encoding each of the two structural domains of the protein, and for the C-terminal domain of the PI 4-kinase. Vectors pREP1 and pREP2 provided selection for leucine or uracil prototrophy, respectively, under the control of the *nmt1* promoter (18). The desired coding regions were cloned into these vectors using PCR for gene amplification and to introduce *Nde*I sites at the initiation codons and *Bam*HI sites immediately 3′ to the termination codons. Plasmid pRep1-cPI4K includes the C-terminal domain of PI 4-kinase that gave a positive interaction with Cdc4p in the two-hybrid assay. As indicated in Fig. 1, pREP1-Cdc4p includes the complete, authentic *cdc4* coding region. pREP1-Cdc4p(N) encodes the N-terminal of Cdc4p (codons 1–72 fused in frame to codons 136–141). pREP1-Cdc4p(C) and pREP2-Cdc4p(C) encode the C-terminal domain (codons 1–3 fused in frame to codons 73–141). The sequences of the inserts in each expression vector were confirmed.

S. pombe strains MD38, MD39, and MD 40 were transformed by electroporation (19) and cultured in Edinburgh minimal medium plus 10 μM thiamine and appropriate supplements and lacking leucine (pREP1 vectors), uracil (pREP2 vectors), or both leucine and uracil (double transformants). After an overnight incubation, cells were recovered by centrifugation, washed in sterile deionized water, and used to inoculate 50–500 ml of the appropriate medium with or without added thiamine. After 24 h, cells were collected by centrifugation, washed, and either resuspended in lysis buffer for protein extraction and ELISA or fixed with formaldehyde/glutaraldehyde for examination by phase contrast and fluorescence microscopy.

Antibodies, ELISA, and Immunoblotting—Rabbit polyclonal antiserum was generated against a 149-amino acid fragment of the C-terminal domain of PI 4-kinase. An *Eco*RI-*Hin*dIII fragment was cloned into pRSETB vector (Invitrogen Corp., SanDiego, CA), fused to a sequence encoding a polyhistidine metal binding tag. *E. coli* strain BL21(λDE3) (Stratagene, La Jolla, CA) was used for transformation and protein expression after induction with isopropyl-1-thio-β-D-galactopyranoside. Cells were lysed with a French pressure cell and the protein fragment purified first by metal chelate chromatography on Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen), according to the manufacturer's instructions. A protein of appropriate size was identified by differential expression in cells transformed with pRSETB vector with and without insert and by the presence of a polyhistidine domain on Western blots. The protein was further purified by SDS-PAGE, recovered by electro-elution (Bio-Rad), and used to generate rabbit antiserum. To reduce nonspecific binding, the rabbit antiserum was incubated with sonicated BL21 cells transformed with pRSETB vector without insert. Antibodies against Cdc4p and Cdc8p were available from previous work in this laboratory (9, 23). Rabbit antibodies against Myo2p were obtained from M. K. Balasubramanian (11). Anti-Gal4 and anti-His tag antibodies, as well as horseradish peroxidase-conjugated secondary antibodies for Western blotting were from commercial sources.

Levels of protein accumulation were analyzed by Western blotting after separation by SDS-PAGE (24) and immunoblotting (25) to Immobillon-P^{SQ} membranes (Millipore). Tricine-SDS-PAGE in 16.5% gels were used for analysis of Cdc4p individual domain expression (26). For Western blots, primary antibodies were used at 1:10,000 dilution, and goat anti-rabbit IgG peroxidase conjugate (Sigma) was used at 1:3,000. Detection was by ECL Western blotting System (Amersham Pharmacia Biotech).

For ELISA, 0.2 μg of purified Cdc4p[1] in 0.1 M sodium bicarbonate was added to 96-well plates (Linbro-Titertek) and evaporated to dryness in a 30 °C incubator. RIA grade bovine serum albumin was then added to each well at a final concentration of 3% (w/v) in TTBS (Tris-buffered saline containing 0.5% Tween 20) for at least 4 h at room temperature. The blocking solution was aspirated, and cell lysates supplemented with bovine serum albumin to a final concentration of 1% (w/v) were added to the Cdc4p-coated wells and incubated at 4 °C overnight. After five washes with TTBS, wells were incubated for 2 h

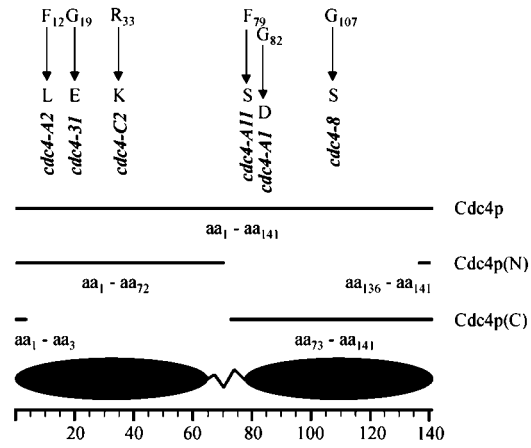


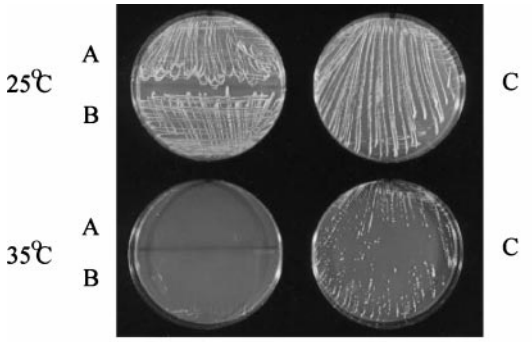
FIG. 1. **Location and identification of point mutations within the primary structure of Cdc4p responsible for temperature-sensitive failure of cytokinesis.** Cdc4p is made of distinct N- and C-terminal domains attached by a flexible linker region (residues 65–78).[1] The residues comprising the two domains and flexible region are indicated schematically as *ovals* and a *broken line*, respectively. The *top part* of the figure shows the amino acid substitution corresponding to a given allele of Cdc4p. For instance, substitution of Phe by Leu (F_{12}) is responsible for the temperature-sensitive phenotype of *S. pombe* cells carrying the *cdc4-A2* allele. Cdc4p, Cdc4p(N), and Cdc4p(C) denote the regions of Cdc4p encoded in the vectors used for ectopic expression in *S. pombe* (pRep vectors) or to test for protein-protein interaction in the yeast two-hybrid assay (pBI880 vectors).

with primary antibodies at 1:1000 dilution in TTBS + bovine serum albumin, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:1000 dilution for 30 min. After further washes, substrates for peroxidase (*o*-phenylenediamine and H_2O_2) were added, and the reactions were terminated exactly 10 min later with sulfuric acid to a final concentration of 0.75 N. Absorbance at 490 nm was measured with a multiwell plate reader. Nonspecific binding of lysate proteins and antibodies was estimated from absorbance in wells not coated with Cdc4p. Cell lysates were prepared by resuspension of cells collected by centrifugation in buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium vanadate, 1% (v/v) Nonidet P40, and protease inhibitor mixture (0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptin and pepstatin, 10 μg/ml aprotinin). The cell suspensions were sonicated for 15–20 min (Biosonik III, Bronwill; at maximum intensity in ice water bath), and the cell lysate supernatants were collected after centrifugation (13,000 × *g*, 15 min) and kept on ice until used. Protein concentration estimation was by the method of Bradford (27), and equivalent amounts of lysate proteins were used in each well.

Microscopy—Cells were fixed with a mixture of glutaraldehyde and formaldehyde, as described by Moreno *et al*. (17). Cell morphology was examined by phase contrast and fluorescence microscopy after staining with Calcofluor White and 4′,6′-diamidino-2-phenylindole dihydrochloride, as described previously (17). Immunofluorescence was also done as described previously with both primary antibodies and Texas red-conjugated secondary antibodies at 1:400 dilution (17). For vizualization of the C-terminal domain of PI 4-kinase, its coding sequence was cloned as an *Nde*I-*Bam*HI insert into pDM212 (provided by Dr. Dan McCollum, Vanderbilt University), a pRep42 vector for expression as a green fluorescent protein fusion, under the control of an attenuated *nmt1* promoter. Transformation and protein expression were carried out as described above. Cells transformed with pDM212 vector without insert served as negative control.

RESULTS

Several Temperature-sensitive Mutant Alleles of cdc4 Show Interallelic Complementation in Diploid Cells—The original genetic description of the *cdc4* locus described two temperature-sensitive lethal mutants (now known as *cdc4-G19E* and *cdc4-G107S;* Fig. 1) that complemented one another yet also recombined at low frequency (8). To confirm that this observation represents interallelic complementation, we constructed the heterozygous diploid strain and, as controls, both homozygous diploid strains. Indeed, the *cdc4-G19E*/*cdc4-G107S* strain



GENOTYPE	VIABILITY 35℃
Homozygous Diploids:	
cdc4-G19E/ cdc4-G19E	No
cdc4-G107S/ cdc4-G107S	No
cdc4-G82D/ cdc4-G82D	No
cdc4-F12L/ cdc4-F12L	No
cdc4-R33K/ cdc4-R33K	Not Det.
Heterozygous Diploids:	
cdc4-G19E/ cdc4-G107S	Yes
cdc4-G19E/ cdc4-G82D	No
cdc4-G19E/ cdc4-F12L	No
cdc4-G19E/ cdc4-R33K	No
cdc4-G107S/ cdc4-G82D	No
cdc4-G107S/ cdc4-F12L	No
cdc4-G107S/ cdc4-R33K	No
cdc4-G82D/ cdc4-F12L	Yes
cdc4-G82D/ cdc4-R33K	No
cdc4-F12L/ cdc4-R33K	Yes

FIG. 2. **Interallelic complementation at the *cdc4* locus.** *S. pombe* diploid strains homozygous or heterozygous for *cdc4* alleles were cultured at temperatures permissive (25 °C) and restrictive (35 °C) for haploid viability. All diploid strains were viable at 25 °C. Three of 10 heterozygous diploids were viable at the restrictive temperature, whereas none of the homozygous diploids grew at 35 °C. The *top panels* show a representative experiment: *cdc4-G107S*/*cdc4-G107S* (*A*), *cdc4-G19E*/*cdc4-G19E* (*B*), and *cdc4-G107S*/*cdc4-G19E* (*C*) are diploid cell colonies after incubation at 25 °C (*upper panel*) or 35 °C (*lower panel*). Note that the heterozygous combination of *cdc4-G107S*/*cdc4-G19E* allowed growth under restrictive conditions. *Not Det.*, not determined.

formed colonies from single cells at either 25 or 35 °C, whereas growth of controls was observed only at 25 °C (Fig. 2, *top panel*). To investigate further the potential for complementation of *cdc4* alleles, diploid *S. pombe* strains carrying heterozygous combinations of other temperature-sensitive alleles were tested for growth at 35 °C. These alleles were obtained from a number of *S. pombe* strains with mutations that mapped tightly to the *cdc4* locus (28). Sequence determination of the Cdc4p coding regions from each strain identified four new alleles, including *cdc4-F12L*, *cdc4-R33K*, *cdc4-F79S*, and *cdc4-G82D*. Fig. 1 shows the single amino acid substitution found in each, as well as the various protein constructs used in this work. The conditionally lethal phenotype of *cdc4-F79S* was incompletely penetrant in haploid cells, and this allele was excluded from further analysis. None of the diploids homozygous for *cdc4-F12L*, *cdc4-G19E*, *cdc4-G82D*, or *cdc4-G107S* grew at 35 °C. Diploid cells homozygous for *cdc4-R33K* were not tested, but given the nature of the screen that led to the isolation of this allele (28), it is likely that cytokinesis is also blocked in this diploid strain. Each of the 10 possible combinations of the five remaining alleles was tested as heterozygous diploid for growth at 35 °C (Fig. 2, *bottom panel*). Of these,

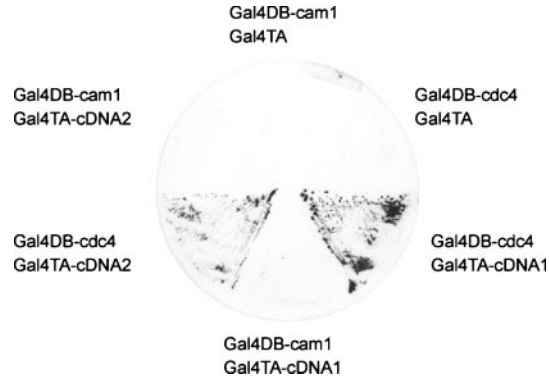


FIG. 3. **The gene products from two distinct *S. pombe* library cDNA clones interact with Cdc4p but not with calmodulin in the yeast two-hybrid assay.** YPB2 cells were cotransformed with *cdc4* (Gal4DB-cdc4) or *cam1* (Gal4DB-cam1) in the bait vector. These strains were cotransformed with each of two library vectors recovered in the screen (Gal4TA-cDNA 1 or 2). Cells were grown on SD-leu-trp-his + 3AT. 3AT-resistant colonies were replica-plated onto nitrocellulose, permeabilized with freeze-thawing in liquid nitrogen, and incubated with reaction mix for β-galactosidase activity (X-gal filter colony assay) (20, 22). Positive interaction is cell growth in the absence of histidine and presence of 3-AT (from *HIS3* gene expression), along with blue color development (*lacZ* expression).

three combinations exhibited complementation. In addition to the originally described combination of *cdc4-G19E* with *cdc4-G107S*, *cdc4-F12L* complemented both *cdc4-G82D* and *cdc4-R33K*. Thus, these results suggest that the two structurally defined N-terminal and C-terminal domains of Cdc4p[1] may be functionally independent.

A Two-hybrid Screen Identified Two Proteins That Interact with Cdc4p, One of Them a Putative Phosphatidylinositol 4-Kinase—One model to explain the complementation described above requires that binding to the first IQ motif of Myo2p may be only one of many functions of Cdc4p. Thus, we searched a cDNA library for sequences encoding other proteins that interact with Cdc4p. A bait vector carrying the coding region of Cdc4p fused to the DNA-binding domain of *Gal4* (*cdc4*/*pBI880*) was introduced into *S. cerevisiae* strain YPB2. The fusion gene was expressed as a 36-kDa protein (predicted molecular mass, 33,000), visualized on Western blots with polyclonal antibodies against either Cdc4p (see Fig. 7) or Gal4p (not shown). The resulting strain was then transformed with vector pBI771 carrying an *S. pombe* cDNA library fused 3′ to the Gal4-*trans*-activator domain coding sequence. From a screen of 3×10^6 cells that contained both bait and library vectors, 12 colonies were recovered that were reproducibly prototrophic for histidine and positive for β-galactosidase activity, our two markers to identify protein-protein interactions. Plasmids carrying each library clone were recovered. The interaction-positive phenotype was confirmed by back transformations of YPB2 cells with the original *cdc4*/*pBI880* vector and each recovered library vector. Additional controls were as follows: YPB2 cells cotransformed with these library vectors and a bait vector without insert or with bait vector carrying the *cdc8* gene instead of *cdc4* failed to grow without histidine. Likewise, YPB2 cells alone or transformed with only the bait vector or any of the library vectors alone were also negative for interaction. Finally, *cdc4* was replaced by calmodulin (*cam1*) in the bait vector and tested for interaction with the library vectors that were positive for interaction with Cdc4p (Fig. 3). Calmodulin is an EF-hand protein about the same size as Cdc4p and 59% similar in primary structure. There was no interaction detected between calmodulin and the library clones that positively interacted with Cdc4p.

Sequence analysis revealed that there were only two distinct

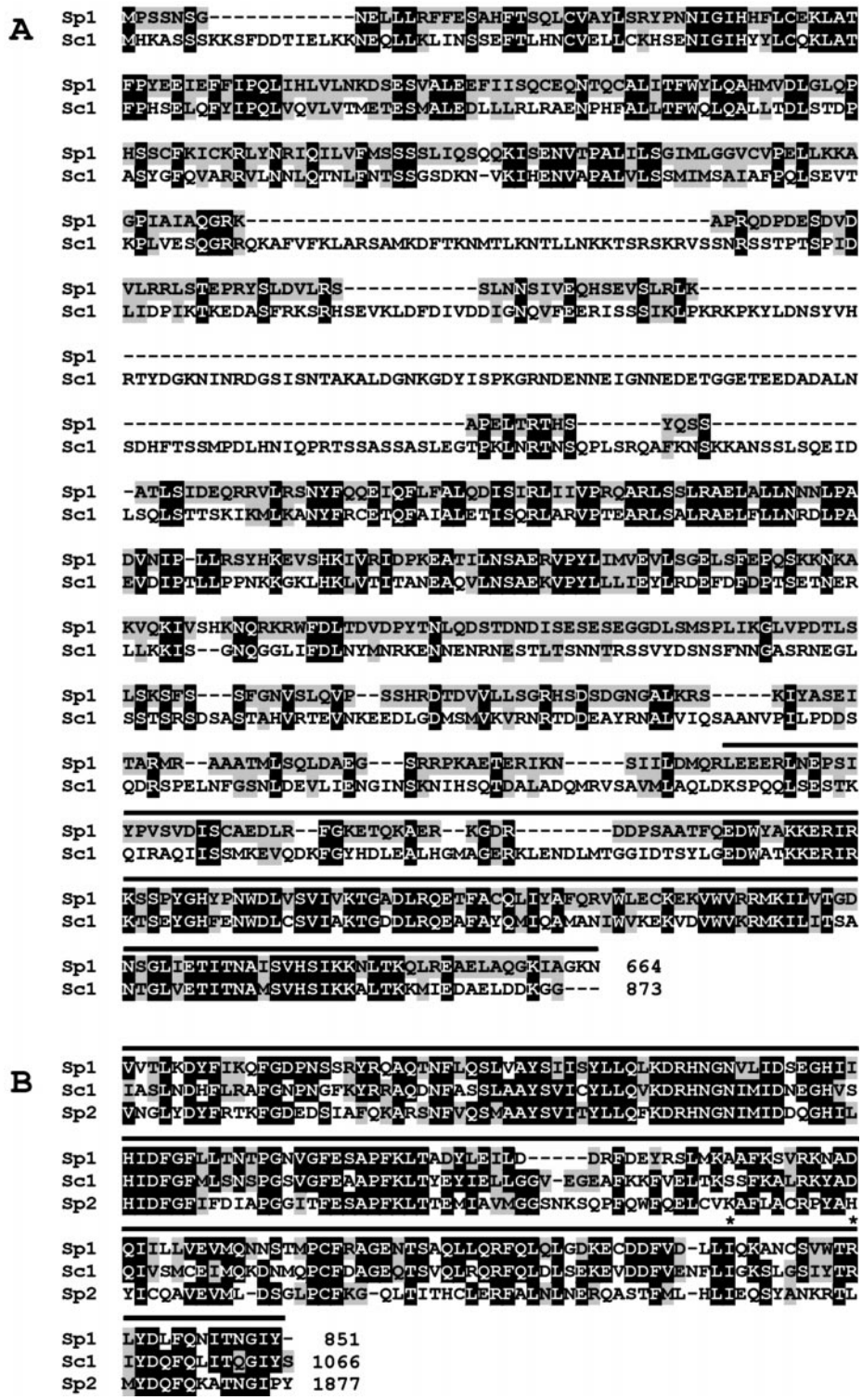


FIG. 4. ***S. pombe*** **phosphatidylinositol 4-kinase peptide sequences aligned with *S. cerevisiae* PIK1 peptide sequence.** The sequence (Sp1) of the *S. pombe* putative PI 4-kinase that was recovered from the two-hybrid screen was aligned with the sequence (Sc1) of the *S. cerevisiae* PIK1. The alignment is presented in two parts: the N-terminal regions (*A*): Sp1, residues 1–664, and Sc1, residues 1–873, and the C-terminal regions (*B*): Sp1, residues 665–851, and Sc1, residues 874–1066. The *solid line* above the sequence indicates the region of Sp1 encoded by the cDNA clone (residues 507–851) recovered from the two-hybrid screen. The Sanger Centre Sequencing program identified and annotated a second, larger *S. pombe* putative PI 4-kinase (Sp2), with a C-terminal region (residues 1687–1877) similar to that of Sp1 and Sc1 (*B*). No significant similarity was detected outside of this latter region between any of the three proteins. *Asterisks* above the Sp1 sequence (residues 828 and 838) mark the extent of a possible IQ motif.

cDNA clones among the 12 recovered. One identical insert of 693 bp was recovered from seven clones, and another insert of 1141 bp was recovered from the other five. The smaller cDNA clone encodes the C-terminal 230 amino acids of a predicted 610-amino acid *S. pombe* protein, identified by the Sanger Center Genome Sequencing Project (TrEMBL accession number O13821). This protein shares some similarity with a zinc finger domain protein in *S. cerevisiae*, Vps27p, that is implicated in vacuolar and endocytic membrane traffic (29). The larger cDNA clone encodes the C-terminal 345 residues of a predicted 851-amino acid *S. pombe* protein, annotated as a phosphatidylinositol 4-kinase by the Sanger Center (SwissProt accession number Q10366). This annotation is based on similarity to the *S. cerevisiae* PIK1 protein, a confirmed phosphatidylinositol 4-kinase (30). The Cdc4p-binding domain of this *S. pombe* PI 4-kinase has 53% amino acid identity with the corresponding C-terminal domain of PIK1, which includes its putative catalytic site (30, 31) as well as 41% amino acid identity with the kinase domain of a much larger PI 4-kinase (a predicted 1877-amino acid protein), recently identified by the Sanger Center in *S. pombe* (TrEMBL accession number Q9USR3) (Fig. 4). There is no significant sequence similarity between these two *S. pombe* proteins outside their kinase domains.

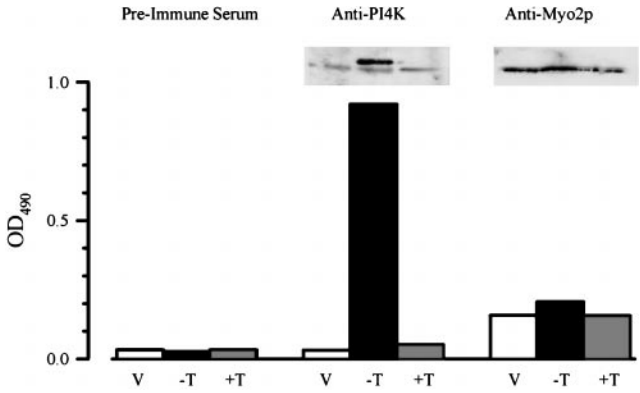


FIG. 5. **Cdc4p binds to both the C-terminal domain of the PI 4-kinase and to Myo2p in immunosorbent assays.** Multiwell plates were coated with purified Cdc4p and incubated with proteins extracts from *S. pombe* cells transformed with pRep1 vector without insert (*V*) or transformed with pRep1 vector carrying the coding sequence of the C-terminal domain of the PI 4-kinase. Cells were grown in the presence (+*T*; repressing) or absence (−*T*; expressing) of thiamine. Cells transformed with vector without insert were grown in the absence of thiamine. Binding of either the C-terminal domain of PI 4-kinase or of Myo2p to Cdc4p-coated wells was estimated with additions of antibodies against PI 4-kinase or Myo2p, followed by horseradish peroxidase-conjugated anti-rabbit IgGs. The *bottom panel* shows the changes in A_{490} 10 min after addition of substrates for horseradish peroxidase. Preimmune serum (collected from the same rabbit prior to immunization with a fragment of PI 4-kinase) was used as control. Nonspecific binding assessed from wells not coated with Cdc4p was subtracted. The results shown are from one of four separate experiments. PI 4-kinase binding to Cdc4p was always enhanced with extracts from cells cultured in the absence (−*T*) compared with the presence (+*T*) of thiamine, with the ELISA signal enhancement varying from 2.7- to 15-fold, between four experiments. The *top panel* shows the identification of proteins recovered from Cdc4p-coated wells by immunoblot analysis. For each sample, proteins present in six Cdc4p-coated wells as described above were recovered in SDS-PAGE buffer after five washes in TTBS, separated by SDS-PAGE, and identified by immunoblotting with anti-PI 4-kinase and anti-Myo2p antibodies. Results indicate the presence of the ectopically expressed C-terminal domain of PI 4-kinase in Cdc4p-coated wells incubated with protein extracts from cells grown in the absence of thiamine (derepressed) only and the presence under all growth conditions of endogenous Myo2p. Results shown are from one of two separate experiments.

Both Myo2p and the C-terminal Domain of the PI 4-Kinase Bind Cdc4p in Immunosorbent Assays—To confirm the interaction observed between Cdc4p and the PI 4-kinase in the yeast two-hybrid assay, as well as the previously reported interactions with Myo2p, we evaluated binding *in vitro* by immunoassays. For these studies, the C-terminal domain of the PI 4-kinase was cloned into vector pRep1 for expression in *S. pombe,* under the control of the *nmt1* (no message in thiamine 1) promoter. The resulting vector is called pRep1-cPI4K. Multiwell plates were coated with purified Cdc4p and incubated with protein extracts prepared from *S. pombe* cells that were transformed with pRep1-cPIK4 and grown in the presence or absence of thiamine or from cells transformed with pREP1 lacking an insert. After extensive washes, binding of candidate proteins to immobilized Cdc4p was evaluated by ELISA with polyclonal antibodies against Myo2p and PI 4-kinase, respectively. As shown in Fig. 5, Cdc4p-coated wells that were treated with *S. pombe* cell extracts showed strong anti-Myo2p immunoreactivity, confirming prior evidence of binding of Myo2p to Cdc4p (11). Note that the Myo2p detected represented the endogeneous protein and that in this experiment similar levels of binding were detected with or without the pRep1-cPI4K vector and in the presence or absence of thiamine. Myo2p was also detected by immunoblotting after proteins were recovered from Cdc4p-coated wells and separated by denaturing polyacrylamide gel electrophoresis. Similar signals were obtained regardless of whether extracts were from cells cultured with or without thiamine or transformed with vector lacking insert.

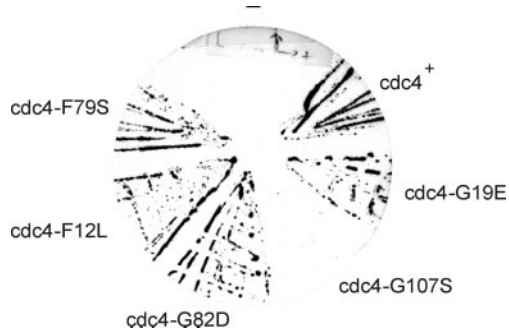


FIG. 6. **Interaction between the PI 4-kinase and *cdc4* mutant alleles.** YPB2 cells cotransformed with the C-terminal domain of PI 4-kinase in the library vector and intact *cdc4* (cdc4$^+$) or mutated alleles of *cdc4* in the bait vector (Fig. 2) were grown on SD-leu-trp-his + 3AT. Negative control (YPB2 cells transformed with the PI 4-kinase in the library vector and with bait vector lacking an insert) is shown at the *top* (−). A positive interaction is shown by growth at 25 °C in the absence of histidine and presence of 3-AT (from *HIS3* gene expression) and by blue color development (*lacZ* expression). Results indicate that a single point mutation, G107S, abolishes the interaction between Cdc4p and the PI 4-kinase.

Strong anti-PI 4-kinase immunoreactivity was observed in Cdc4p-coated wells that were treated with protein extracts from cells expressing the C-terminal domain of the PI 4-kinase. PI 4-kinase binding to Cdc4p was always higher with extracts from cells transformed with pRep1-cPI4K and cultured without thiamine, compared with cells grown with thiamine. A protein of the size predicted for the C-terminal domain of the PI 4-kinase (35 kDa) was recovered from wells that had been coated with Cdc4p and incubated with protein extracts from cells cultured in the absence of thiamine (Fig. 5, *top panels*). No such protein was detected when extracts were from cells grown in the presence of thiamine or when the cells were transformed with the pRep1 vector lacking insert. Taken together, these assays show binding between Cdc4p and the same C-terminal domain of PI 4-kinase that was positive for interaction in the two-hybrid assay. There was no significant immunoreactivity when preimmune serum replaced the anti-PI 4-kinase or anti-Myo2p antibodies. Likewise, no significant immunoreactivity was observed with antibodies against Cdc8p (not shown). The latter is also in accord with our two-hybrid assay, which did not show interaction between Cdc8p and Cdc4p.

A Single Amino Acid Substitution, G107S, Abolishes the Interaction between Cdc4p and the C-terminal Domain of the PI 4-Kinase—To assess the specificity of the interaction between Cdc4p and the PI 4-kinase in the two-hybrid assay and its possible physiological relevance, we introduced into the bait vector each of the *cdc4* alleles known to produce temperature-sensitive failure of cytokinesis in *S. pombe*. Single base pair substitutions were introduced into bait vector pBI880 to generate genes encoding Gal4DB-Cdc4p fusion proteins with the predicted single amino acid substitutions F12L, G19E, F79S, G82D, and G107S. YPB2 cells were cotransformed with each bait vector carrying a mutated *cdc4* gene and with prey vector pBI771 encoding the C-terminal domain of PI 4-kinase. Cotransformation with bait vector lacking an insert was a negative control, whereas cotransformation with wild-type *cdc4* in pBI880 was a positive control. YPB2 cells carrying pBI880 vectors with intact or mutated *cdc4* genes accumulated the expected fusion proteins as judged by immunoblotting (not shown). As expected, intact Cdc4p showed a positive interaction with the C-terminal domain of PI 4-kinase (Fig. 6). None of the single point mutations, F12L, G19E, F79S, or G82D, impaired the ability of Cdc4p to interact with the PI 4-kinase (Fig.

6). In contrast, Cdc4p-G107S did *not* interact, indicating that substitution of Gly^{107} with Ser selectively impaired the ability of Cdc4p to bind the PI 4-kinase. Thus, there is a site-specific interaction between Cdc4p and the C-terminal domain of PI 4-kinase that likely involves Gly^{107}, a residue that is known to be important for Cdc4p function.

In parallel experiments, the C-terminal domain of the Vps27p-like protein showed positive interactions with both wild-type Cdc4p and with Cdc4p-G107S (not shown), indicating that the latter mutant protein was competent to sustain this specific two-hybrid interaction, although it was not competent for interaction with the PI 4-kinase. The Cdc4p-Vps27p-like protein interaction was also maintained by Cdc4p carrying substitutions, G19E, G82D, and F79S, but not with Cdc4p-F12L (not shown), indicating different structural requirements for binding of Cdc4p to the PI 4-kinase and the Vps27p-like protein.

The C-terminal Domain of Cdc4p Interacts with the PI 4-Kinase in the Two-hybrid Assay—Cdc4p has two structurally distinct domains, defined approximately by the N-terminal and C-terminal halves of the polypeptide.[1] Because Gly^{107} is located in the C-terminal domain, it is possible that this domain is able to interact with the PI 4-kinase autonomously. To test this hypothesis, partial coding regions of the *cdc4* gene were introduced into vector pBI880. Based on the tertiary structure of Cdc4p in solution, the N-terminal domain included the first 72 amino acids terminating in the interdomain linker region (Cdc4p(N)), whereas the C-terminal domain included the last 68 amino acids (Cdc4p(C)) (Fig. 1). Both constructs also retained the coding regions for the first three and last five amino acids of Cdc4p. All three fusion proteins (Gal4DB-Cdc4p, Gal4DB-Cdc4p(C), and Gal4DB-Cdc4p(N)) accumulated in transformed YPB2 cells, as detected on anti-Cdc4p immunoblots (Fig. 7, *top panel*). Both intact Cdc4p and the C-terminal domain of Cdc4p showed positive interaction with the PI 4-kinase, whereas the N-terminal domain did not (Fig. 7, *bottom panel*). Thus, the C-terminal domain alone is sufficient to establish the interaction between Cdc4p and the PI 4-kinase in the two-hybrid assay. In contrast, neither the C-terminal domain nor the N-terminal domain of Cdc4p alone interacted with the Vps27p-like protein (not shown).

Intact Cdc4p Is Required for Cytokinesis—Based on this study, the interaction between Cdc4p and PI 4-kinase appears to be limited to the C-terminal domain of Cdc4p. Therefore, we asked whether the presence of the wild-type C-terminal domain on its own was sufficient to restore cytokinesis function to cells bearing mutations in the corresponding region of Cdc4p. Also, we noted that in two of the three observed cases of interallelic complementation one allele is mutated in the N-terminal domain and the other in the C-terminal domain (Figs. 1 and 2). Thus, the basis for the complementation, in these cases, might be that each structural domain functions independently of the other. Therefore, recombinant genes were constructed for expression of either or both of the structural domains of Cdc4p in *S. pombe* strains containing temperature-sensitive *cdc4* alleles. The same *cdc4* coding regions used in the yeast two-hybrid assay were introduced into pRep1 for expression under the control of the *nmt1* promoter.

Wild-type cells transformed with pRep1-Cdc4p were cultured in the presence or absence of thiamine, and protein extracts were prepared and assayed for levels of Cdc4p by immunoblotting. In the presence of thiamine, the abundance of Cdc4p was not elevated above the normal level, which was at the detection limit of this assay (Fig. 8, *lane 4+* compared with *lanes 1+*, *2+*, and *3+*). Derepression of the plasmid-borne *cdc4* gene resulted in markedly increased Cdc4p levels (Fig. 8, *lanes 4*, + and −). In cells cultured at 25 °C in the absence of thiamine, the increased abundance of Cdc4p had no apparent effect on growth or morphology of wild-type cells or of cells carrying the *cdc4-G19E* or *cdc4-G107S* allele. However, both *cdc4-G107S* cells and *cdc4-G19E* cells transformed with pRep1-Cdc4p were viable and grew at 35 °C when cultured without thiamine (not shown). Thus, overexpression of the wild-type gene rescued the temperature-sensitive lethal phenotype of the two mutant alleles.

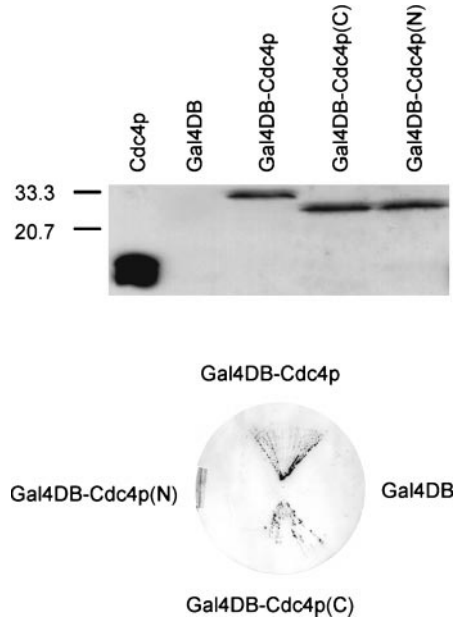


FIG. 7. **The C-terminal domain of Cdc4p alone is sufficient to establish an interaction with the PI 4-kinase.** Intact Cdc4p, as well as the C- and N-terminal domains of Cdc4p (Fig. 1), were cloned in the bait vector and tested for interaction with the PI 4-kinase, as described in Fig. 6. All Gal4DB fusion proteins of the appropriate sizes accumulated in YPB2 cells, as shown in immunoblots with anti-Cdc4p antibodies (*top panel*). The *bottom panel* shows the results of the X-gal filter colony assay. There is positive interaction in YPB2 cells cotransformed with the library vector carrying the coding sequence of the C-terminal domain of PI 4-kinase and with the bait vector carrying the coding sequence of either Cdc4p (Gal4DB-Cdc4p) or of its C-terminal domain (Gal4DB-Cdc4p(C)). There is no interaction in cells transformed with bait vector without insert (Gal4DB) or carrying the coding sequence of the N-terminal domain of Cdc4p (Gal4DB-Cdc4p(N)).

Wild-type cells transformed with pRep1-Cdc4p(N) were assayed for protein accumulation as above. The N-terminal domain was not detected after growth in the presence of thiamine. However, under derepressing conditions it accumulated to an apparently high level (Fig. 8, *lanes 1*, + and −). Transformation with pRep1-Cdc4p(N) had no apparent effect on the growth or morphology of wild-type cells, *cdc4-G107S* cells or *cdc4-G19E* cells cultured under derepressing conditions at 25 °C (not shown). Thus, accumulation of high levels of the N-terminal domain of Cdc4p did not appear to be deleterious in wild-type or mutant genetic backgrounds. Neither *cdc4-G19E* nor *cdc4-G107S* cells were rescued for growth at 35 °C by expression of the N-terminal domain gene alone (not shown). Thus, accumulation of this isolated N-terminal domain containing a wild-type glycine at position 19 did not provide the function(s) lost in the *cdc4-G19E* or *cdc4-G107S* mutant.

The C-terminal domain was not detected in protein extracts from wild-type cells transformed with pRep2-Cdc4p(C) and cultured without thiamine (Fig. 8, *lane 2−*). Coexpression of the N-terminal domain did not change this result (Fig. 8, *lane 3−*). The polyclonal serum used in this assay is known to detect each domain of Cdc4p with similar sensitivity (Fig. 7). We are confident that the SDS-PAGE system used resolves the C-

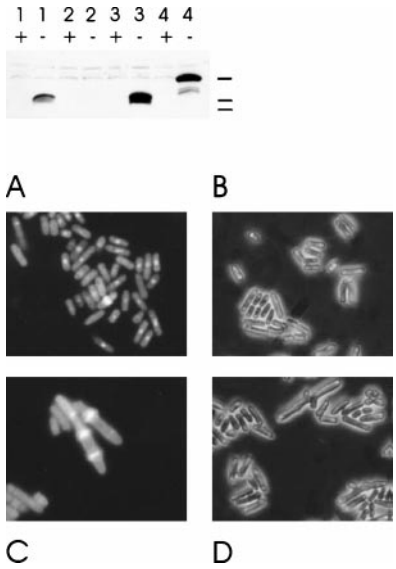


FIG. 8. **Effects of ectopic expression of Cdc4p and its N- and C-terminal domains in wild-type and Cdc4p mutant allele strains of *S. pombe*.** Regions of Cdc4p encoded by vectors pRep1-Cdc4p, pRep1-Cdc4p(N), and pRep2-Cdc4p(C) are shown in Fig. 1. *Top panel*, immunoblot analysis of protein extracts from *S. pombe* cells probed with anti-Cdc4p polyclonal antibodies. Cells transformed with pRep1-Cdc4p(N) (*lane 1*), pRep2-Cdc4p(C) (*lane 2*), pRep1-Cdc4p(N) and pRep2-Cdc4p(C) (*lane 3*), pRep1-Cdc4p (*lane 4*), and cultured for 24 h, under repressed (+ 10 μg/ml thiamine) or derepressed (− thiamine) conditions. *Horizontal lines* to the *right* indicate the expected positions of Cdc4p (*top line*), Cdc4p(N) (*middle line*), and Cdc4p(C) (*bottom line*). Note the accumulation of Cdc4p and Cdc4p(N) but not Cdc4p(C) under derepressed conditions. *Bottom panel*, effects of expressing the Cdc4p C-terminal domain on the morphology of wild-type cells (calcofluor-stained, *A*; phase-contrast, *B*) and of cells carrying the *cdc4-G107S* allele (calcofluor-stained, *B*; phase contrast, *D*). Note the elongated cell phenotype with multiple septum typical of abnormal cytokinesis in cdc4-G107S cells derepressed for expression of the C-terminal domain of Cdc4p.

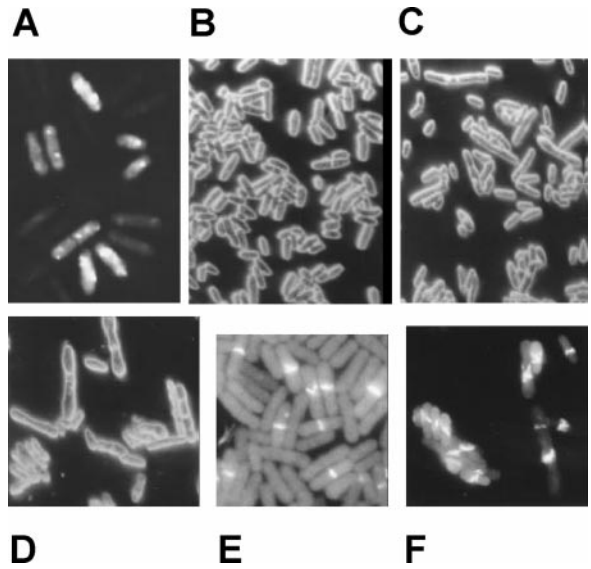


FIG. 9. **Localization and effects of ectopic expression of the C-terminal domain of the PI 4-kinase on *S. pombe* morphology.** *A*, the C-terminal domain of PI 4-kinase fused to the green fluorescent protein is not found at the medial band but in dots near one or both poles of the cell. *B*, gross morphology assessed by phase contrast microscopy is normal in *S. pombe* cells with wild-type *cdc4* grown in the absence of thiamine (derepressed). *C*, morphology of *cdc4-G107S* cells grown in the presence of thiamine appears to be normal. *D*, in contrast, morphology of *cdc4-G107S* cells grown in the absence of thiamine is aberrant because of expression of the C-terminal domain of the PI 4-kinase. *E*, Calcofluor White staining shows some abnormalities in septum material deposition in *S. pombe* cells with wild-type *cdc4* grown in the absence of thiamine. *F*, these abnormalities are most severe in *cdc4-G107S* cells grown under the same conditions, that is, at 25 °C in the absence of thiamine.

terminal domain and of its migration relative to that of the N-terminal domain because we have produced and analyzed two fragments that correspond closely to Cdc4p(N) and Cdc4p(C) by cleavage of purified Cdc4p at the acid-sensitive site in the linker region (Asp75/Pro76) (not shown). Thus, the absence of the N-terminal domain and/or some part of the interlinker region appears to destabilize the C-terminal domain of Cdc4p, presumably toward proteolytic degradation. Not surprisingly, therefore, in the absence of significant accumulation under derepressed conditions, *cdc4-G107S* cells transformed with pRep2-Cdc4p(C) were not viable at 35 °C (not shown). Likewise, coexpression of the separate C- and N-terminal domains failed to rescue the growth defect of either *cdc4-G19E* or *cdc4-G107S* cells at 35 °C (not shown).

In summary, only the intact Cdc4p protein was able to provide function in *S. pombe* cytokinesis. Expression of the N-terminal domain alone did not rescue the growth defects of the *cdc4-G19E* cells, and the C-terminal domain appears unstable in its absence.

Ectopic Expression of the Cdc4p C-terminal Domain or the Cdc4p-binding Domain of the PI 4-Kinase Affects Cytokinesis—Ectopic expression of Cdc4p(C) did not affect the growth or morphology of wild-type cells (Fig. 8). Similarly, *cdc4-G19E* cells appeared to be unaffected (not shown). However, growth of *cdc4-G107S* cells at the permissive temperature was sensitive to derepression of the *nmt1* promoter. 24 h after shifting to medium lacking thiamine at 25 °C, most *cdc4-G107S* cells carrying pRep2-Cdc4p(C) were elongated, multinucleate, and multiseptate (Fig. 8). This phenotype was not observed of *cdc4-G107S* cells carrying pRep1-Cdc4p or pRep1-Cdc4p(N). Thus, expression of the gene encoding Cdc4p(C) produced an allele-specific, cell cycle phenotype that affected cytokinesis. This observation also suggests that the C-terminal domain of Cdc4p (or a proteolytic fragment of it) accumulated to some level and possessed biological activity despite the fact that it was not detectable on immunoblots (Fig. 8).

To investigate the possible colocalization of the PI 4-kinase and Cdc4p, a gene encoding GFP fused to the N terminus of the Cdc4p-binding domain of the PI 4-kinase was expressed in *S. pombe* under control of a weakened *nmt1* promoter. Examination of these cells revealed punctuate fluorescence and often a large dot near one or both poles of the cell (Fig. 9). Fluorescence was not observed as a medial band.

The biological relevance of the interaction between Cdc4p and the PI 4-kinase was also evaluated by monitoring the effects of ectopic expression of the kinase on cytokinesis. The C-terminal domain of the PI 4-kinase was expressed under control of the *nmt1* promoter in wild-type cells for *cdc4* or carrying alleles *cdc4-G19E* or *cdc4-G107S*. The cell content of Cdc4p was not affected (not shown). Cells were examined for growth characteristics, cell morphology, presence of septa, and distribution of some contractile ring proteins. When the C-terminal domain of the PI 4-kinase was expressed in cells with wild-type background, there were no obvious growth or gross morphological effects (Fig. 9). Cells appeared unaffected in size, with a normal distribution of mononuclear and binuclear cells. However, calcofluor staining revealed that a small proportion of cells carried septa that were abnormal in appearance (Fig. 9), suggesting some disruption in septum formation and possibly in contractile ring formation. Cells exhibiting abnormal septum staining were otherwise morphologically unremarkable. Indirect immunofluorescence microscopy with antibodies specific for Myo2p or Cdc8p revealed apparent contractile ring

staining consistent with the pattern observed for septum staining (not shown). That is, a proportion of cells exhibited abnormal medial staining, suggesting that contractile ring formation or stability was impaired. In contrast, abnormal septum morphology was not observed when cells were grown in the presence of thiamine, which repressed expression of the C-terminal domain of PI 4-kinase. Similar results were observed at 25 °C in cells bearing the *cdc4-G19E* mutant allele (not shown).

In contrast, expression of the C-terminal domain of PI 4-kinase had pronounced effects in cells bearing the *cdc4-G107S* allele. Cell viability was reduced, and the majority of cells were morphologically highly abnormal (Fig. 9). Effects included cell elongation, branching, as well as the presence of multiple nuclei and septa. In particular, there were pronounced defects in septum formation and persistence of primary septum material (Fig. 9). The distributions of Cdc4p, Cdc8p, and Myo2p, assessed by indirect fluorescence microscopy, each showed aberrant medial staining patterns (not shown), in keeping with the abnormal calcofluor staining. Cells with the *cdc4-G107S* allele grown in the presence of thiamine exhibited greatly reduced abnormalities (Fig. 9).

Thus, ectopic expression of the C-terminal domain of PI 4-kinase affected formation or stability of the contractile ring with this effect being most pronounced in cells bearing the G107S allele of *cdc4*. As discussed below, these results suggest that interaction between Cdc4p and the C-terminal domain of PI 4-kinase observed first in the yeast two-hybrid system and then in immunosorbent assays also occurs in intact cells, disrupting the formation or stability of the contractile ring.

DISCUSSION

Is the Sole Function of Cdc4p That of a Myosin Essential Light Chain?—There is general acceptance that some form of sliding filament mechanism is responsible for force production by the contractile ring (2, 3, 7). The ring is made of actin, one or possibly two myosins (Myo2p and Myp2), a tropomyosin (Cdc8p), and presumably two light chains, one of which is Cdc4p (5, 9, 12, 13, 23, 28, 32). Although intact myosin has yet to be purified from *S. pombe*, there is genetic, biochemical, and structural evidence in favor of a role for Cdc4p as a myosin essential light chain (9, 11).[1] In muscles, the sole established function of essential light chains is to provide structural support for the neck region of the heavy chain of myosin. No additional roles for the ELC are known. However, a contractile ring is distinct from muscles in many respects, and a direct analogy does not satisfactorily explain many characteristics of cytokinesis. For instance, there is complex interaction of the contractile ring with the cell membrane that must be flexible enough to allow changes as the cell circumference decreases during division (7). There is rapid assembly and dissassembly of the ring that must be coordinated in time and location with other events in the cell cycle (2–4). In *S. pombe,* contractile ring function must be coordinated also with formation and dissolution of septum material that composes the rigid outer wall of the organism. Finally, the need for variable contractile speed and force development, hallmarks of muscle function, is not an obvious requirement of cytokinesis.

Our results suggest that Cdc4p is more than a myosin essential light chain in *S. pombe*. First, it is very difficult to explain the observed interallelic complementation if the function of Cdc4p is strictly that of an essential light chain, and second, we found two proteins that also interact with Cdc4p, in addition to Myo2p. Interallelic complementation is usually observed when a protein has more than one independent essential function, or alternatively, when mutant forms of the protein can assemble into an active oligomeric complex. Thus, as discussed in detail by Slupsky *et al*.,[1] one model to explain the interallelic complementation could be that Cdc4p interacts with both IQ domains of myosin playing essentially the structural roles of both essential and regulatory light chains. The key features of such a model would be that two *cdc4* proteins bind to the neck region of the myosin heavy chain and that both Cdc4p-Cdc4p and Cdc4p-Myo2p interactions are essential for function. Structural and dynamic studies of Cdc4p by NMR spectroscopy indicate that the protein is flexible enough that it could bind to myosin at either or both of the two IQ domains in a manner analogous to that observed in the muscle myosin-ELC-RLC complex (10). This is also consistent with the observation that the two domains of Cdc4p are not functionally independent in that only expression of intact Cdc4p can rescue the cytokinesis defects observed in yeast with mutant alleles (Fig. 8). However, there is another small EF-hand protein with sequence similarity to known regulatory light chains, Rlc1p, that binds to Myo2p at the IQ motif corresponding to an RLC-binding site.[3] Interallelic complementation would then be possible if Cdc4p and Rlc1 can bind to myosin interchangeably or at different times in the formation or function of the ring to allow for Cdc4p:Cdc4p interaction. The stoichiometry of association and binding affinities of Cdc4p and Rlc1p with Myo2p and/or Myp2p are unknown.

Cdc4p Interacts with a PI 4-Kinase—Another possibility to explain the interallelic complementation, which is not mutually exclusive to that described above, is that Cdc4p has two or more essential functions. Prior studies involving synthetic lethal interactions or suppressor mutation approaches have identified genetic interactions involving *cdc4* (14, 33, 34), However, such studies do not provide direct evidence for interaction between two proteins. This is the first study to employ a yeast two-hybrid screen of an *S. pombe* cDNA library. Using this approach, we identified a Vps27p-like protein and a putative PI 4-kinase that interact with Cdc4p. Three sets of observations further confirm the initial finding of a biologically relevant interaction between Cdc4p and PI 4-kinase: (i) the two-hybrid interaction is domain-specific and suppressed by one mutation in that domain that is also responsible for temperature-sensitive failure of cytokinesis; (ii) there is direct evidence of interaction in ELISA; and (iii) ectopic expression of the Cdc4p-binding domain of PI 4-kinase causes allele-specific failure of cytokinesis.

The interaction phenotype in the yeast two-hybrid assay was strong, reproducible, and specific. For instance, calmodulin, another EF-hand protein with sequence and structural similarity to Cdc4p did not interact with the PI 4-kinase (Fig. 3). Furthermore, the interaction was abolished by a single amino acid substitution in the C-terminal domain of Cdc4p, G107S, known to produce a temperature-sensitive defect in cytokinesis in *S. pombe* (Fig. 6). This effect was specific to the interaction with the PI 4-kinase, because Cdc4p-G107S was still able to bind the Vps27p-like protein, whereas, conversely, the F12L mutation in the N-terminal domain of Cdc4p disrupted the latter but not the former interaction. In fact, the C-terminal domain of Cdc4p alone is sufficient to interact with the PI 4-kinase in the two-hybrid assay (Fig. 7). Again, this is different from the interaction between Cdc4p and the Vps27p-like protein, where the N-terminal domain of Cdc4p alone failed to interact. Thus, some interactions of Cdc4p require the intact molecule, whereas others may be domain-specific.

Interaction between Cdc4p and the C-terminal domain of PI 4-kinase was confirmed directly with *in vitro* binding assays (Fig. 5). Both endogenous Myo2p and the ectopically expressed C-terminal domain of PI 4-kinase were bound to immobilized Cdc4p, as detected in enzyme-linked immunosorbent assays. Examination of the sequence of this PI 4-kinase domain indi-

cates that there may be a poorly conserved IQ motif starting at residue 828. This is a candidate binding site for Cdc4p, similar to that found in Myo2p (11). In some but not all experiments, there was also a reduction in the apparent level of binding of endogenous Myo2p to Cdc4p in cells expressing the C-terminal domain of PI 4-kinase (not shown). It is tempting to speculate that this resulted from a competition between Myo2p and the C-terminal domain of PI 4-kinase for binding to Cdc4p. However, this might have been caused by variable level of expression of the C-terminal domain of PI 4-kinase between experiments or by differences in efficiency of extraction/solubilization of either or both of the PI 4-kinase or Myo2p. In addition there was weak but measurable immunoreactivity with anti-PI 4-kinase antibodies when immobilized Cdc4p was incubated with extracts from wild-type cells. This is probably due to presence of the intact PI 4-kinase (predicted molecular mass, 96,657) in the protein extracts because our antiserum can recognize a large protein in whole cell extracts (not shown). However, this protein appears to be present at low concentration, and it may not have been appropriately solubilized by our extraction protocols. Attempts to clone the full-length PI 4-kinase in our expression vectors were not successful.

Evidence for a significant *in vivo* interaction between Cdc4p and the PI 4-kinase is provided by the observation that ectopic expression of the C-terminal domain of the PI-4-kinase affects contractile ring formation and cell morphology. Overexpression of a Cdc4p-binding protein in *S. pombe* would be expected to disrupt cytokinesis, presumably by titrating out or sequestering Cdc4p from its proper site of interaction. Alternatively, because the C-terminal domain of the PI 4-kinase also appears to contain a putative catalytic domain, disruption of cell function may result from its aberrant location or activity. Expression of the C-terminal domain of the PI 4-kinase resulted in disruption in the formation or stability of the contractile ring that was most severe in cells bearing the G107S mutant allele of Cdc4p (Fig. 9). These cells had markedly reduced growth and a morphology characteristic of failure of cytokinesis, namely, elongated cells with multiple nuclei and abnormal septa. The actin ring serves to guide and position the septum in *S. pombe*. Accumulation of disorganized septum material in the medial region of the cell is indicative of defects in contractile ring assembly or stability (3, 4, 6, 28). Abnormal septum morphology was observed in many cells bearing the wild-type or the G19E allele of Cdc4p but without overall effects on cell growth and morphology. These effects were specific to expression of the C-terminal domain of the PI 4-kinase because in identical experiments, expression of the Vps27p-like protein, which also interacts with Cdc4p in the yeast two-hybrid assay, had no effect on cell growth and morphology.

The severity of the disrupting effects of overexpressing the C-terminal domain of PI 4-kinase in *cdc4-G107S* cells is at first surprising. That is, because the G107S mutation abolished interaction of Cdc4p with the PI 4-kinase in the two-hybrid assay, it seems unlikely that Cdc4p is simply being sequestered from the contractile ring by overexpression of the kinase. However, we cannot exclude the possibility of a weak interaction between Cdc4p-G107S and the PI 4-kinase, undetectable in the two-hybrid assay, that is sufficient to disrupt cytokinesis in intact cells. Alternatively, it may be that ectopic expression of the C-terminal domain of the PI 4-kinase is disrupting interactions with other proteins involved in cytokinesis that are also weakened in function by the conditional mutation in Cdc4p. Regardless, our results suggest there is a potentially important role for a PI 4-kinase in an aspect of cytokinesis, that is also dependent upon Cdc4p. Thus, combining ectopic expression of the C-terminal domain of the kinase with a mutation in Cdc4p produces a synthetic cell cycle phenotype.

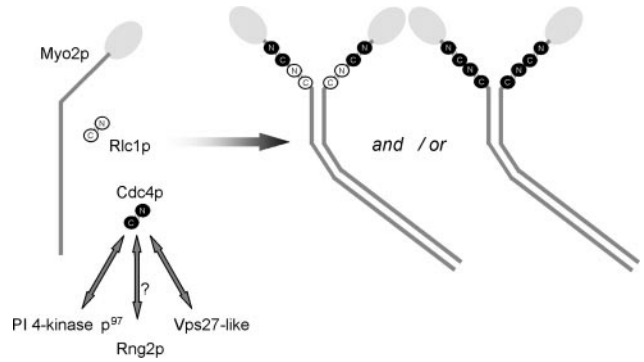


FIG. 10. **Known and hypothesized interactions involving Cdc4p.**

Some Functional Considerations—At first glance, it appears surprising that a postulated myosin light chain interacts with a protein involved in membrane traffic, the Vps27p-like protein, and a PI 4-kinase. However, unlike muscles, a contractile ring is a dynamic structure changing in composition and appearance throughout cytokinesis. It is possible that Cdc4p is playing an early role in the assembly of the ring, as postulated previously (4), and a later one in its function as a myosin light chain. Its function may be somewhat akin to that of a calmodulin, which interacts with many proteins, including myosins. The interaction of Cdc4p with a PI 4-kinase may be particularly relevant to early recruitment of ring components. In addition to their role in signaling through catalyzing the phosphorylation of phosphatidylinositol, PI 4-kinases are involved in the formation of lipid-protein interactions with cytoskeletal proteins (31, 35). Many actin-binding proteins that bind to phosphorylated phosphoinositides participate in rearrangement of the actin cytoskeleton (35). PIK1, the closest relative to *S. pombe* PI 4-kinase, is essential for cytokinesis in *S. cerevisiae* (30). Disruptions of the PIK1 gene are lethal, whereas temperature-sensitive mutant alleles of PIK1 are defective in cytokinesis (30). Although most PI 4-kinases are cytosolic, one report suggests that PIK1 is associated with the nucleus. This led to the suggestion that the enzyme may function to link completion of nuclear division with cytokinesis (30). Another PI 4-kinase, the product of the *Stt4* gene, is required to delay cytokinesis until the mitotic spindle is properly positioned, playing a part in a postulated cytokinesis checkpoint in *S. cerevisiae* (36). Although the cellular location and precise activity of the PI 4-kinase identified herein remains to be established, the observation that related kinases have a role in cytokinesis provides compelling functional support for a biological role of the interaction with Cdc4p detected by two-hybrid screens and immunoassays.

In summary, we suggest that an interaction between Cdc4p and a PI 4-kinase likely contributes to recruitment and/or assembly of some of the many cytoskeletal proteins required in the formation and function of the contractile ring. How this interaction takes place or is regulated is uncertain. Cdc4p is phosphorylated *in vivo*, but its phosphorylation is not essential, because mutations of two critical serine residues to aspartic acid did not affect the ability of the cells to grow or divide (37). Likewise, binding of calcium to Cdc4p is unlikely to modulate its interaction with target proteins. Cdc4p has one (out of the four) EF-hand motifs with the appropriate side chains for metal chelation, but does not bind calcium *in vitro*.[1] Overall, Cdc4p appears to play a multi-faceted role in cytokinesis. In addition to serving at least as a myosin essential light chain within the contractile ring, Cdc4p interacts both genetically

and directly with several additional proteins, one of which is a putative PI 4-kinase (Fig. 10).

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Cdc4p, a Contractile Ring Protein Essential for Cytokinesis in *Schizosaccharomyces pombe* , Interacts with a Phosphatidylinositol 4-Kinase

Michel Desautels, Jason P. Den Haese, Carolyn M. Slupsky, Lawrence P. McIntosh and Sean M. Hemmingsen

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