

Structure of Cdc4p, a Contractile Ring Protein Essential for Cytokinesis in *Schizosaccharomyces pombe**

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The *Schizosaccharomyces pombe* Cdc4 protein is required for the formation and function of the contractile ring, presumably acting as a myosin light chain. By using NMR spectroscopy, we demonstrate that purified Cdc4p is a monomeric protein with two structurally independent domains, each exhibiting a fold reminiscent of the EF-hand class of calcium-binding proteins. Although Cdc4p has one potentially functional calcium-binding site, it does not bind calcium *in vitro*. Three variants of Cdc4p containing single point mutations responsible for temperature-sensitive arrest of the cell cycle at cytokinesis (Gly-19 to Glu, Gly-82 to Asp, and Gly-107 to Ser) were also characterized by NMR and circular dichroism spectroscopy. In each case, the amino acid substitution only leads to small perturbations in the conformation of the protein. Furthermore, thermal unfolding studies indicate that, like wild-type Cdc4p, the three mutant forms are all extremely stable, remaining completely folded at temperatures significantly above those causing failure of cytokinesis in intact cells. Therefore, the altered phenotype must arise directly from a disruption of the function of Cdc4p rather than indirectly through a disruption of its overall structure. Several mutant alleles of Cdc4p also show interallelic complementation in diploid cells. This phenomenon can be explained if Cdc4p has more than one essential function or, alternatively, if two mutant proteins assemble to form a functional complex. Based on the structure of Cdc4p, possible models for interallelic complementation including interactions with partner proteins and the formation of a myosin complex with Cdc4p fulfilling the role of both an essential and regulatory light chain are proposed.

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The atomic coordinates and structure factors (code 1GGW) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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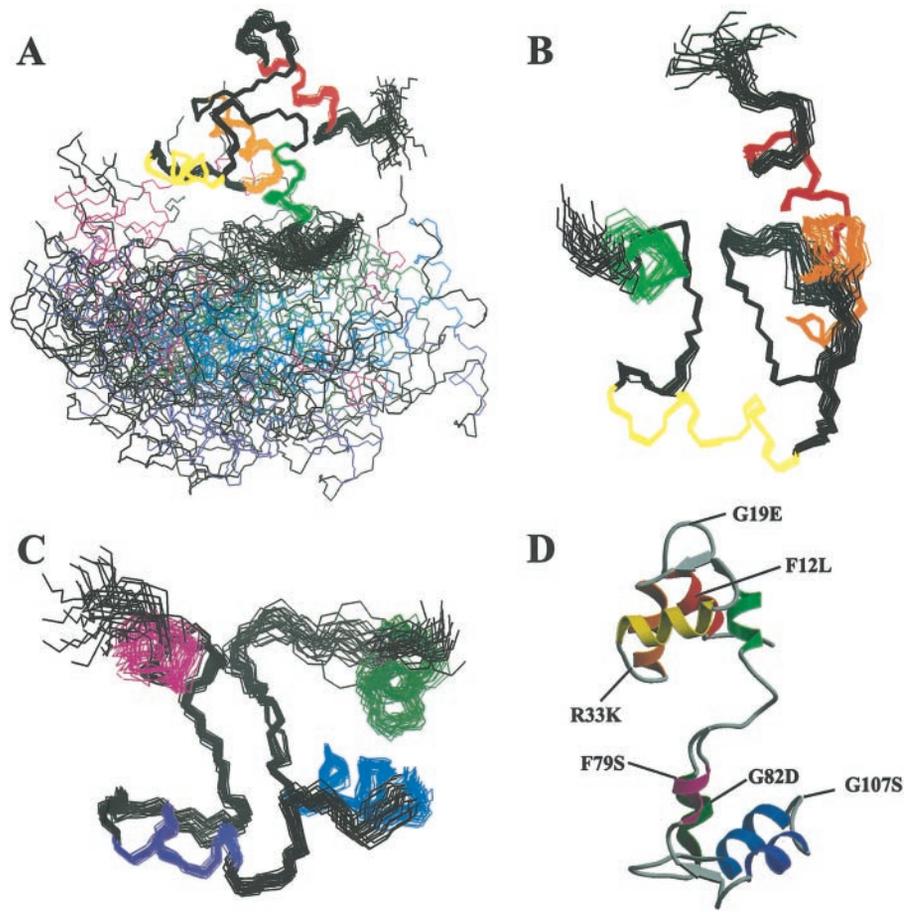
Cytokinesis begins during anaphase and completes shortly after mitosis. Prior to this event, a contractile ring containing actin and myosin forms at the medial plane of the cell. The diameter of this ring decreases progressively during cytokinesis, presumably due to contractile forces generated by the myosin motor, and thereby results in the division of one parent cell into two daughter cells (for review see Refs. 1 and 2). Although the contractile ring is a complex structure, it is highly amenable to molecular genetic studies. Well characterized genes encoding some of the main cytoskeletal components essential for cytokinesis include tropomyosin (*cdc8*) (3), actin (*act1*) (4), a putative myosin light chain (*cdc4*) (5, 6), and two myosins (*myo2* and *myp2*) (7–9). The product of the *myo2* (or *rng5*) gene is essential for contractile ring function, whereas that of the *myp2* (or *myo3*, *myo22*) gene appears required only under certain conditions (9). Both Myo2p and Myp2p are type II myosins and thus are predicted to consist of two heavy chains that dimerize via an extended C-terminal coiled-coil domain. The globular N-terminal domain of each heavy chain is separated from this coiled-coil by a flexible neck region. In conventional myosins, such as those found in muscle, two EF-hand proteins, termed the essential light chain (ELC)¹ and regulatory light chain (RLC), associate with the N-terminal and C-terminal halves of each neck region, respectively, at IQ-motifs (IQXXXXRXXXXR, where X is any residue) (10, 11). By analogy, we might also expect an ELC and an RLC to bind each heavy chain in *Schizosaccharomyces pombe*, playing a structural support role for the α -helical neck region of myosin.

The *cdc4* locus, which was identified in the original screens for cell division control mutants, is required for septum formation and cell separation (12). Cells with conditionally lethal point mutations become elongated, dumbbell-shaped, multinucleate and fail to divide before dying. A similar phenotype is observed when the *cdc4* gene is disrupted (5). It was suggested that Cdc4p is a myosin light chain based on sequence similarities with EF-hand proteins, on localization to the contractile ring, and on association with Myo2p (5, 13).

Although very likely a myosin light chain, the sequence of Cdc4p is sufficiently distinct that it is not possible to confidently predict whether it functions as the structural equivalent of an ELC or RLC. Site-directed mutation of a conserved resi-

¹ The abbreviations used are: ELC, essential light chain; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum correlation; RLC, regulatory light chain; 4-kinase, phosphatidylinositol 4-kinase; cTnC cardiac troponin C; r.m.s., root mean square; mutations are designated by the single letter codes for the wild-type and mutant sequences, respectively, separated by the residue number.

FIG. 1. Cdc4p is composed of two structurally distinct domains connected by a flexible linker. Shown is the ensemble of 26 structures calculated for the wild-type protein, superimposed using the backbone atoms in the α -helices of the N-terminal domain (A, all residues; B, residues 2–66 only), and the C-terminal domain (C, residue 77–141 only). Due to the flexible linker, the N- and C-terminal domains do not have a fixed orientation with respect to one another. A MOLSCRIPT ribbon diagram of one representative structure of Cdc4p is shown in D, with α -helices colored as in A–C and β -strands indicated as white arrows. Helix boundaries are as follows: A (8–14), red, B (26–35), orange, C (41–49), yellow, D (58–64), green, E (79–86), green, F (96–105), blue, G (113–119), purple, and H (133–137), magenta. The short antiparallel β -sheets encompass residues 22–24 and 54–56 in the N-terminal domain and 93–95 and 127–129 in the C-terminal domain. Also indicated in D are the positions of point mutations in the N- (F12L, G19E, and R33K) and C-terminal domains (F79S, G82D, and G107S) causing temperature-dependent cell growth arrest at cytokinesis. Serines 2 and 6, which are sites of phosphorylation *in vivo* (40), lie at the exposed N terminus of the protein.



due in the IQ motif proximal to the Myo2p N terminus results in loss of association with Cdc4p in immunoprecipitation assays (13). This indicates that Cdc4p associates with Myo2p at least at the equivalent of an ELC-binding site (14). However, *S. pombe* cells expressing only the mutated form of Myo2p were still viable, suggesting that some binding of Cdc4p may occur *in vivo* (13). Alternatively, Cdc4p may also interact with other proteins in the contractile ring, besides Myo2p, such as Myp2p or Rng2p (7, 9, 15). Recently, a gene that presumably encodes a small EF-hand protein was annotated as a putative RLC by the Sanger Centre Genome Project (accession number CAB54151). This RLC-like protein also localizes to the contractile ring and appears to interact with Myo2p at the second IQ motif, a recognized binding site for RLC in conventional myosins.² Thus, current evidence suggests that Myo2p is a conventional myosin with Cdc4p serving as an ELC and possibly a second related protein as an RLC.

However, the report that initially identified the *cdc4* locus also described a pair of closely linked *cdc*⁻ mutants that complemented one another and recombined with low frequency (12). This interallelic complementation, which cannot be explained if the only role of Cdc4p is to serve as an ELC, might arise if Cdc4p has two independent, essential functions, each selectively disrupted by a single mutation. Indeed, as shown in the accompanying paper (49), Cdc4p interacts with several additional proteins besides Myo2p, including a putative phosphatidylinositol 4-kinase. Alternatively, interallelic complementation can occur if two mutant forms of a protein assemble to form a functional oligomeric complex in a diploid cell. In support of this latter case, an attractive structural model can be developed in which two mutant forms of Cdc4p, serving as

both an ELC and RLC, bind to myosin at both IQ motifs and physically interact with one another via their respective wild-type surfaces.

A major goal of our research is to understand the structural basis for the essential functions of Cdc4p, with particular emphasis on defining how conditional mutants of Cdc4p fail to complete cell division under restrictive conditions and on explaining the observed interallelic complementation of these mutants. There are currently no structural data available for any of the cytoskeletal proteins involved in the mechanism and regulation of cytokinesis. To this end, we have used NMR spectroscopy to determine the tertiary structure of wild-type Cdc4p and to characterize three temperature-sensitive mutants of this protein. In parallel with the accompanying paper (49) describing the identification by genetic and immunochemical methods of proteins that interact with Cdc4p, we discuss possible models for the function of this essential component of the *S. pombe* cytokinesis machinery.

MATERIALS AND METHODS

Labeling and Purification of Cdc4p for NMR Spectroscopy—A *cdc4* cDNA construct in the *Escherichia coli* expression vector pRSET B (Invitrogen Corp., San Diego, CA) was a gift of Dr. Dan McCollum (Vanderbilt University). Vectors for expression of *cdc4* temperature-sensitive mutants were made by cloning polymerase chain reaction-amplified coding regions of *cdc4-G19E*, *cdc4-G82D*, and *cdc4-G107S* into pT7-7 (16). All sequences were confirmed using a model 370A automated sequencer (PE Applied Biosystems Inc.).

E. coli strains BL21(λ DE3) (Stratagene, La Jolla, CA) and CT19 (*avtA::Tn5/trpB83::Tn10/dcm ompT lon 8DE3 ilvE12 tyrB507 aspC13*) (17) were transformed with the appropriate expression vector immediately prior to use. Unlabeled, uniformly ¹⁵N- and ¹³C-labeled and selectively α -¹⁵N-labeled proteins were prepared using media as described previously (18). After induction with 1 mM isopropyl-1-thio- β -D-galactopyranoside and growth overnight at 25–30 °C, *E. coli* cells were har-

² M. K. Balasubramanian, personal communication.

TABLE I
Structural statistics

R.M.S.D. from the average structure (\AA^2) ^a	
N-terminal domain	C-terminal domain
Backbone atoms, 0.41 ± 0.07	Backbone atoms, 0.71 ± 0.11
Heavy atoms, 0.90 ± 0.07	Heavy atoms, 1.21 ± 0.13
NOE restraints ^b	
Total, 1056	Total, 925
Intra-residue, 511	Intra-residue, 480
Sequential, 259	Sequential, 239
Medium range, ^c 168	Medium range, ^c 138
Long range, 118	Long range, 68
H bond restraints, 24	H bond restraints, 38
Dihedral restraints ^b	
Total, 120	Total, 94
ϕ , 44	ϕ , 39
ψ , 49	ψ , 39
χ_1 , 27	χ_1 , 16
Energy	
<i>kcal/mol</i>	
E_{total} , 171.1 ± 5.2	
E_{NOE} , 15.4 ± 1.8	
E_{CDIH} , 2.1 ± 0.3	
E_{VDW} , 8.2 ± 1.8	
$E_{\text{L-J}}$, -610.3 ± 15.6	
R.M.S.D. from idealized geometry	
Bond lengths (\AA), 0.0038 ± 0.0001	
Bond angles ($^\circ$), 0.42 ± 0.01	
Improper ($^\circ$), 0.22 ± 0.01	

^a 32 structures were used in the final calculation. Because the central linker is unstructured, root mean square deviations (R.M.S.D.) were calculated for each domain separately (residues 8–64 for the N-terminal domain and 79–137 for the C-terminal domain).

^b The force constants for the calculation of NOE and dihedral energies were 50 and 200 kcal/mol, respectively. The structures all satisfy experimental restraints with no distance violations greater than 0.3 \AA and no dihedral violations greater than 5 $^\circ$.

^c ($2 \leq |i - j| \leq 4$).

^d The force constant for calculation of the van der Waals energy (F_{repel}) was $0.75 \text{ kcal mol}^{-1} \text{\AA}^{-4}$.

vested by centrifugation and suspended in 30 ml of Buffer A (30 mM Tris-HCl, pH 7.5, 2 mM 2-mercaptoethanol, 15% glycerol) per 500 ml of culture. Cells were lysed using a French pressure cell, followed by centrifugation. The supernatant was loaded onto a Fractogel anion exchange column (Merck), which was developed at 3 ml/min in Buffer A using a linear NaCl gradient from 0 to 600 mM. SDS-polyacrylamide gel electrophoresis analysis showed that Cdc4p eluted from the column between 300 and 400 mM NaCl. Protein from these fractions was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (70% saturation) and dissolved in Buffer A containing 150 mM NaCl. The protein solution was loaded onto a Superdex 75 gel filtration column (Amersham Pharmacia Biotech), and fractions were collected at 1 ml/min. The Cdc4p was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (70% saturation), dissolved in 25 mM NH_4HCO_3 , pH 8.5, and loaded on a Superdex 75 column equilibrated with 25 mM NH_4HCO_3 for buffer exchange. The final fractions were pooled, concentrated in Centricon-3 microconcentration device (Amicon Inc., Beverly, MA), and lyophilized in a Speed-Vac. The identity of each protein was confirmed by mass spectrometry. During preparation of the protein, the N-terminal methionine was removed by *E. coli* proteases.

NMR Spectroscopy—NMR experiments were performed on ~5 mM wild-type or mutant Cdc4p dissolved in 100 mM KCl, 1 mM EDTA, 8 mM dithiothreitol, pH 6.5, in either 90% H_2O , 10% D_2O or 100% D_2O . All data were recorded at 30 $^\circ\text{C}$ with a Varian Unity 500-MHz NMR spectrometer equipped with a pulsed-field gradient accessory. NMR data were processed using NMR pipe (19) and analyzed using the program PIPP (20). Essentially complete ^1H , ^{13}C , and ^{15}N spectral assignment of wild-type Cdc4p was obtained using an extensive set of gradient-enhanced three-dimensional experiments as outlined in Ref. 21. The diastereotopic methyl groups of valine and leucine were stereospecifically assigned using biosynthetically directed ^{13}C labeling (22). Both histidine residues in Cdc4p were shown to be partially deprotonated and

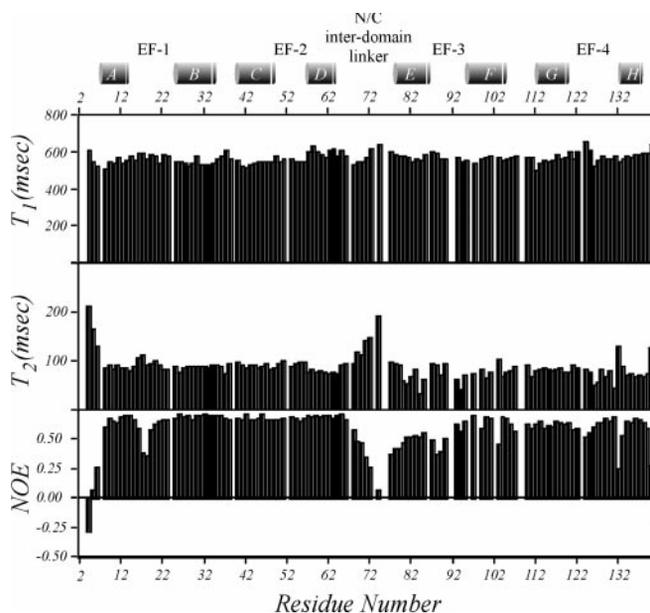


FIG. 2. Plot of backbone amide ^{15}N T_1 , T_2 , and heteronuclear $^{15}\text{N}(^1\text{H})$ -NOE values versus residue number for wild-type Cdc4p. The N and C termini and the linker region connecting the two domains of the protein exhibit conformational flexibility on a sub-nanosecond time scale as indicated by anomalously high T_2 and low heteronuclear NOE values. Residues within the loop regions, particularly between helices A and B and helices E and F, also show evidence of conformational mobility. The positions of the eight helices are indicated above the figure with cylinders.

predominantly in the N^{H} tautomeric form at pH 6.5 using the HMBC experiment (23). Assignments of the ^1H and ^{15}N resonances in uniformly ^{15}N -labeled Cdc4p-G19E, Cdc4p-G82D, and Cdc4p-G107S were obtained using three-dimensional ^{15}N -TOCSY/NOESY-HSQC experiments. To confirm these assignments, HSQC spectra were also recorded on Cdc4p-G107S selectively labeled with ^{15}N -Phe and ^{15}N -Leu, and on Cdc4p-G82D selectively labeled with ^{15}N -Phe, ^{15}N -Leu, ^{15}N -Val, and ^{15}N -Ala. ^{15}N T_1 , T_2 , and heteronuclear $^{15}\text{N}(^1\text{H})$ -NOE relaxation data were recorded and analyzed on uniformly ^{15}N -labeled wild-type and mutant proteins, as described (24).

Three-dimensional structures were computed from experimental restraints starting with an extended chain using a simulated annealing protocol with X-PLOR version 3.8 (25). Experimental distance restraints were obtained as described (26–28) using three-dimensional ^{15}N -NOESY HSQC, three-dimensional simultaneous $^{13}\text{C}/^{15}\text{N}$ NOESY-HSQC, four-dimensional $^{13}\text{C}/^{13}\text{C}$ HMQC-NOESY-HMQC spectra, and a two-dimensional homonuclear NOESY experiment in D_2O (for NOEs involving aromatic protons), all recorded with a $\tau_{\text{mix}} = 75$ ms. Hydrogen bonds were included as distance restraints for those amides remaining protonated 60 min after transfer of the protein into D_2O buffer. Torsion angle ϕ restraints were obtained from an analysis of an HNHA experiment (29) and ψ restraints from an analysis of the $d_{\text{N}\alpha}/d_{\text{C}\alpha\text{N}}$ ratio according to (30). χ_1 angles were restrained according to a staggered rotamer model using coupling patterns observed for stereospecifically assigned $\text{H}\beta, \beta'$ in ^{15}N -TOCSY-HSQC and HNHB spectra and for the methyls of Thr, Ile, and Val in long range $^{13}\text{C}\gamma$ - ^{15}N and $^{-13}\text{C}'$ correlation spectra (29).

Circular Dichroism Spectroscopy—The circular dichroism spectra of ~10 μM wild-type or mutant Cdc4p in 30 mM potassium phosphate buffer, pH 6.5, were acquired with a Jasco J-730 CD spectropolarimeter using a 0.1-cm water-jacketed quartz cell. Thermal denaturation curves were recorded by monitoring the signal at 222 nm as a function temperature, increased at 1 $^\circ\text{C}/\text{min}$.

RESULTS

Cdc4p Is Made of Two Distinct Domains Joined by a Flexible Linker Region

The tertiary structure of Cdc4p was determined from 2195 NMR-derived restraints using a simulated annealing protocol

(Fig. 1A).³ The ensemble of 26 calculated structures exhibit good covalent geometry as indicated by low r.m.s. deviations from idealized values and by low NOE, dihedral angle, and van der Waals energies (Table I). For all 26 structures, 99.3% of the main chain (ϕ , ψ) angles fall in the core or allowed regions of the Ramachandran map, as determined using PROCHECK-NMR (31). With the exception of the N and C termini (residues 2–7 and 138–141, respectively) and a central linker region (residues 65–78), the structures of the backbone and core side chain atoms of Cdc4p are well defined by NMR data.

As shown in Fig. 1D, Cdc4p adopts a dumbbell-shaped structure with distinct N and C domains. Each consists of four α -helices, named A through D in the N domain (Fig. 1B) and E through H in the C domain (Fig. 1C), and a short two-stranded anti-parallel β -sheet. Joining the two domains is a proline-rich linker region (residues 66–77) that is devoid of any regular structure. Based on ¹⁵N NMR relaxation measurements (see below), the linker is highly flexible in solution, and thus the N- and C-terminal domains do not have a fixed orientation with respect to one another. Consistent with this conclusion, Cdc4p can be chemically cleaved into two separate domains, and each domain can be expressed in isolation (49). The monomeric nature of Cdc4p is indicated by the narrow resonances detected within its NMR spectra and confirmed by both heteronuclear relaxation measurements, which reveal ¹⁵N T_1 and T_2 values consistent with a protein of ~15 kDa total molecular mass, and by equilibrium ultracentrifugation runs which indicated an average molecular mass of 14.1 ± 0.9 kDa over three iterations at 15,000 rpm (not shown).

Cdc4p Contains Four EF-hand Motifs but Does Not Bind Calcium

Consistent with predictions based on sequence alignments, Cdc4p contains four EF-hand structural motifs (32), defined by helices A/B and C/D in the N-terminal domain (Fig. 1B) and by E/F and G/H in the C-terminal domain (Fig. 1C). The two EF-hands within each domain are joined by the antiparallel pairing of the short β -strands located within the loop regions between their constituent helices.

The structural similarity between Cdc4p and other EF-hand proteins such as calmodulin prompted us to investigate the possibility of calcium binding by Cdc4p. Accordingly, ¹H-¹⁵N HSQC NMR spectra of Cdc4p were recorded both in the presence of excess calcium and of excess EDTA. The NMR spectrum of a protein is extremely sensitive to structural and electrostatic perturbations and thus serves as an excellent indicator of ligand binding. The complete lack of any significant spectral changes under either condition (data not shown) strongly suggests that Cdc4p does not bind calcium with any appreciable affinity.

Dynamic Properties of Cdc4p

¹⁵N NMR relaxation measurements were carried out to investigate the dynamic properties of the backbone of Cdc4p. As shown in Fig. 2, residues at the N and C termini and within the linker region connecting the two domains of the protein exhibit long transverse (T_2) relaxation times and reduced heteronuclear ¹⁵N{¹H}-NOE values compared with those in regions of well defined secondary structure. These results are diagnostic of fast internal motions relative to the global tumbling of the protein (33). Therefore, the apparent disorder or high r.m.s. deviations seen for these regions within the ensemble of structures calculated for Cdc4p (Fig. 1A) can be attributed to con-

formational flexibility on the sub-nanosecond time scale. The dynamic disorder of the termini and linker are further supported by the observation that backbone amides within these regions do not show any significant protection from hydrogen-deuterium exchange with solvent (data not shown). Together, these measurements confirm that the N- and C-domains of Cdc4p are independent structural units tethered by a flexible proline-rich linker sequence.

¹⁵N NMR relaxation measurements also indicate the loop regions in Cdc4p are flexible on a sub-nanosecond time scale. Slightly longer transverse (T_2) relaxation times and reduced heteronuclear ¹⁵N{¹H}-NOE values for amides in the loop regions of the EF-hands I, III, and IV (residues 16–19, 87–90, and 121–126), as well as the loop regions between the EF-hands in the two domains (residues 36–40 and 106–112), indicate more mobility than seen for amides forming elements of regular secondary structure. This corresponds to the slightly higher r.m.s. deviations observed for these loop residues in the ensemble of calculated structure (Fig. 1, B and C). Not surprisingly, the dynamic properties of the backbone of Cdc4p are similar to that observed for other EF-hand proteins, such as apo-cTnC (34).

It is interesting to note that, although both domains of Cdc4p are of similar size, the backbone root mean square deviations from the average structure for the C-domain are almost twice that of the N-domain (Table I). This is a direct result of the measurement of fewer dihedral and medium/long range inter-proton distance restraints in the C-domain *versus* the N-domain. However, we attribute this to different motional properties of the domains of Cdc4p rather than poorer spectral resolution for residues in the C-domain. This conclusion is based on two related observations. First, peaks corresponding to several residues in the C-domain in the ¹H-¹⁵N-HSQC NMR spectrum of Cdc4p exhibit weak intensity (Gly-82, Gln-84, Val-85, Phe-86, Met-93, Ile-94, Gly-95, Asn-112, Asp-125, Val-129, Tyr-131, and His-132), suggestive of a conformational exchange phenomenon occurring on a sub-millisecond time scale. Second, based on ¹⁵N NMR relaxation measurements, the average ¹⁵N T_1 and T_2 values for the N-domain (residues 8–64) are 557 ± 27 and 87.8 ± 7.3 ms, respectively, whereas those for the C-domain (residues 79–137) are 566 ± 26 and 75.6 ± 16.5 ms, respectively (Fig. 2). In particular, the shorter T_2 values, along with a greater deviation from the average, for amides in the C-domain of Cdc4p also suggest that the backbone of this portion of the protein may undergo slow motions that could lead to line broadening. In addition, the average heteronuclear ¹⁵N{¹H}-NOE values for the N- and C-domains are 0.67 ± 0.06 and 0.58 ± 0.09 , respectively. The slightly lower NOE values for amides in the C-domain suggest that the residues may also be under rapid internal motions on a sub-nanosecond time scale. A similar pattern of complex relaxation behavior was observed in a ¹⁵N NMR relaxation study of calmodulin (35).

Structural and Dynamic Properties of Cdc4p Temperature-sensitive Mutants

Three conditionally lethal, loss-of-function point mutations in Cdc4p were studied using ¹H-¹⁵N NMR spectroscopy. The positions of these mutations (G19E, G82D, and G107S) are mapped on the structure of the wild-type protein in Fig. 1D. To investigate the structural and dynamic effects of these amino acid substitutions, three approaches were taken. First, ¹H^N and ¹⁵N chemical shift differences between the wild-type and mutant proteins were measured as a sensitive indicator of structural perturbations (Fig. 3). Second, ³J_{HN-H α} coupling constants were measured to investigate possible changes in backbone ϕ dihedral angles. Finally, ¹⁵N relaxation measurements

³ The coordinates for the ensemble of structures, along with experimental restraints and NMR chemical shifts, have been submitted to the Protein Data Bank and the BioMagResBank.

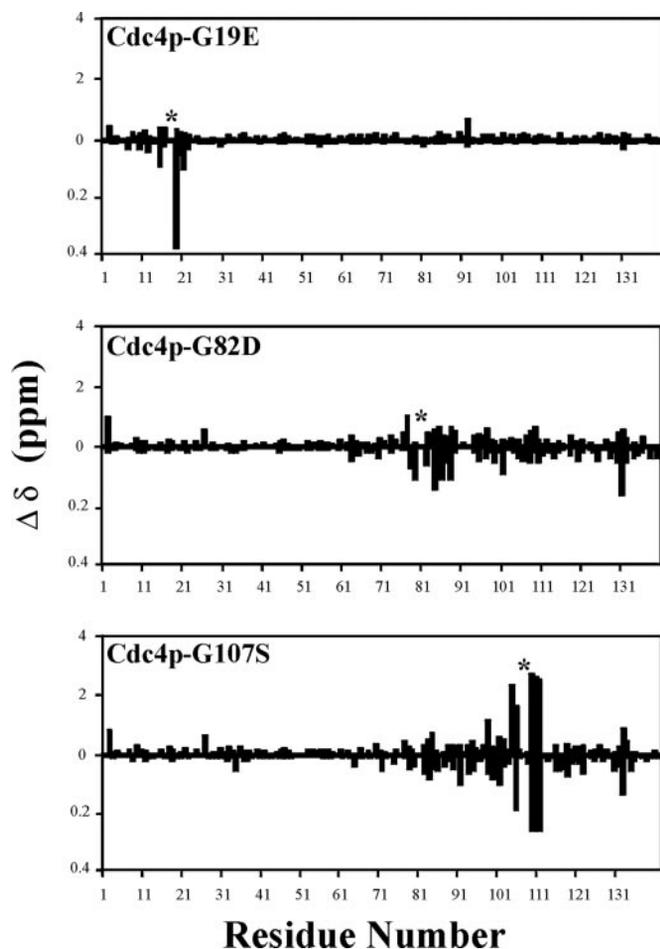


FIG. 3. Amide chemical shift perturbations indicate that only small structural perturbations result from three amino acid substitutions that lead to a temperature-dependent phenotype for Cdc4p. Shown are plots of the absolute values of the chemical shift differences between the ^{15}N (upwards bars) and ^1H (downwards bars) chemical shifts of corresponding amides in each mutant protein versus wild-type Cdc4p. The position of each amino acid substitution is indicated with an asterisk. Small chemical shift differences (<0.03 ppm in ^1H and <0.25 ppm in ^{15}N), as well as changes near His-131, are attributed to slight variations in sample buffer conditions.

were utilized to probe changes in the dynamic behavior of the amide residues in the backbone of the protein. As discussed below, these measurements demonstrated that each of the mutant forms of Cdc4p adopts a stable, folded native-like conformation at pH 6.5 and 30 °C, with only small structural and dynamic perturbations occurring within the domain of the Cdc4p containing the site of substitution.

Cdc4p-G19E—This glycine to glutamic acid mutation occurs in the exposed loop region of the first EF-hand (Fig. 1D). Although the wild-type (ϕ , ψ) angles would disfavor a non-glycine residue, any structural perturbations resulting from the substitution appear small as evident by the localized chemical shift changes (Fig. 3) and the lack of significant changes in the $^3J_{\text{HN-H}\alpha}$ coupling constants. It is interesting to note that the region of the wild-type protein near position 19 has relatively high root mean square deviations in the ensemble of calculated structures (Fig. 1), as well as anomalous ^{15}N T_2 and heteronuclear NOE values (Fig. 2), both of which are indicative of local conformational mobility. The ^{15}N relaxation parameters of Cdc4p-G19E also remained similar to those of the wild-type protein. Therefore, it is likely that Cdc4p readily accommodates a glutamic acid residue at this surface position without significantly altering its structure or dynamic properties.

Cdc4p-G82D—This glycine to aspartic acid mutation falls

within the middle of helix E (Fig. 1D). The chemical shifts of amides throughout the linker and the entire C-domain are perturbed, suggesting that small structural changes occur throughout the second half of the protein in response to the amino acid substitution. Furthermore, based on changes in the measured $^3J_{\text{HN-H}\alpha}$ coupling constants for residue 87 at the C terminus of helix E (10 to 4 Hz) and residue 96 at the N terminus of helix F (2 to 10 Hz), it appears that the backbone ϕ angles have shifted such that helix E is lengthened and helix F shortened. It is likely that these structural changes, which may reflect a slight rotation of helix E, are necessary to displace the aspartic acid side chain from the hydrophobic interface between helices E and F. Relaxation measurements also reveal that the ^{15}N T_2 lifetimes of residues 72, 77, and 78 are increased as compared with wild-type, indicating greater flexibility in the linker region of the G82D mutant.

Cdc4p-G107S—This glycine to serine mutation occurs within the exposed loop between the 2 EF-hands of the C-domain of Cdc4p (Fig. 1D). Chemical shift perturbations due to the amino acid substitution are most pronounced for amides near the site of the mutation and smaller throughout the rest of the C-domain. In contrast, no significant differences in $^3J_{\text{HN-H}\alpha}$ coupling constants or ^{15}N relaxation parameters were detected between the wild-type and mutant protein (Fig. 3). Together, these data suggest that subtle structural perturbations arise throughout the C-domain in response to the amino acid substitution. These perturbations may result from a change in the conformation of the loop region due the introduction of a side chain at position 107 and thus an alteration in the packing of helices G and H.

Folding Studies of Cdc4p Temperature-sensitive Mutants

NMR measurements clearly reveal the three Cdc4p mutants adopt a folded, native-like structure. Therefore, the temperature-sensitive phenotypes associated with each mutation are unlikely to result from a significant change in the tertiary structure of the protein. This is consistent with cell viability under permissive conditions. To investigate the possibility that the temperature-sensitive phenotypes arise from destabilization of Cdc4p, circular dichroism spectroscopy was used to monitor the thermal unfolding transitions of the purified Cdc4p variants (data not shown). Cdc4p as well as Cdc4p-G19E, -G82D, and -G107S are all extremely stable, remaining completely folded at >70 °C. To obtain measurable unfolding transitions, 4 M urea was added as a denaturant to the sample buffer. Under these conditions, all four proteins exhibited broad, biphasic thermal denaturation curves, suggesting that the N- and C-domains of Cdc4p unfolded independently and at different temperatures. Wild-type Cdc4p and both Cdc4p-G82D and -G107S exhibited apparent mid-point unfolding temperatures of ~ 50 °C in 4 M urea at pH 7.0, whereas that of Cdc4p-G19E was reduced to ~ 41 °C. Further deconvolution of the data to yield distinct denaturation transitions was not feasible. Regardless, these results strongly suggest that each of the three Cdc4p mutants adopts very stable, native-like folded structures and that the temperature-sensitive phenotypes must arise directly from a disruption of the function of Cdc4p, rather than indirectly through a disruption of its overall structure.

DISCUSSION

Cdc4p Is a Two Domain, EF-hand Protein That Does Not Bind Calcium—Cdc4p is the first component of the *S. pombe* contractile ring to have its tertiary structure determined. As expected from sequence comparisons (5), the basic fold of Cdc4p is that of a dumbbell-shaped protein with two independent domains connected by a flexible linker. Although each domain

TABLE II
Interhelical angles in selected EF-hand proteins

Protein Data Bank accession codes are as follows: 1WDC for the crystal structure of the ELC and RLC bound to myosin, 1CFD for the NMR structure of apocalmodulin (CaM-*apo*), and 1OSA for the crystal structure of calcium-saturated calmodulin (CaM-4Ca²⁺). The coordinates for calcium-saturated cardiac troponin C (cTnC-3Ca²⁺) were a kind gift of Dr. Brian Sykes.

	N-terminal domain		C-terminal domain	
	A/B	C/D	E/F	G/H
Cdc4p	141 ± 5	108 ± 3	133 ± 9	90 ± 5
ELC	136	138	117	91
RLC	94	94	106	105
CaM- <i>apo</i>	134	128	129	132
CaM-4Ca ²⁺	90	86	102	89
cTnC-3Ca ²⁺	137	108	115	115

contains two EF-hand motifs, only the third has the appropriate side chains for metal chelation (36). However, Cdc4p does not bind calcium *in vitro* as evident by the lack of any NMR spectral changes upon addition of excess CaCl₂ or EDTA. Similarly, the essential light chain (ELC) from chicken skeletal muscle has only one potential metal-binding site (EF-hand 3) but also does not bind this metal ion (10, 37). As summarized in Table II, Cdc4p differs in its precise tertiary structure from several well characterized EF-hand proteins, such as calmodulin, troponin C, and the ELC or RLC bound to myosin. These differences undoubtedly reflect the diversity of functions served by this large family of structural and regulatory proteins (for review see Refs. 38 and 39).

Cdc4p as an Essential Light Chain—Structural, biochemical, and genetic evidence suggest strongly that a primary function of Cdc4p is that of an essential light chain. This evidence includes the following: (i) similarity of sequence, structure, and lack of calcium binding with well characterized ELCs; (ii) direct interaction with the contractile ring myosin, Myo2p, as demonstrated by coimmunoprecipitation assays (13) and immunosorbent assays (49); and (iii) disruption of this interaction *in vitro* by mutation of the first IQ motif of Myo2p (13), which corresponds to an established ELC-binding site in conventional myosins.

By analogy to the structure of a scallop muscle ELC-RLC-myosin heavy chain headpiece ternary complex determined by x-ray crystallography (37, 41, 42), the C-terminal domain of Cdc4p most likely interacts with the first IQ motif in Myo2p. However, as indicated in Table II, the interhelical angles within the EF-hand motifs of free Cdc4p differ from those observed for the muscle ELC bound to myosin. In particular, in the crystalline scallop ternary complex, the ELC is anchored tightly to the myosin headpiece via its C-terminal domain, which adopts a “semi-open” conformation, and more weakly via its N-terminal domain in a “closed” conformation (10, 41). (The “open” conformation for EF-hand proteins is defined by an interhelical angle close to 90° (perpendicular), whereas the closed conformation is characterized by an interhelical angle closer to 180° (anti-parallel) (38)) In contrast, both N- and C-domains of Cdc4p in solution exhibit rather mixed conformations, reminiscent of that of calcium-loaded cTnC. This comparison implies that Cdc4p must undergo a conformational rearrangement upon binding to Myo2p. Such conformational changes are well characterized for the association of many EF-hand proteins with their target polypeptides and often serve as a mechanism for the regulation of their activities, *e.g.* via the binding of ligands such as calcium. Dynamic studies of Cdc4p by ¹⁵N relaxation suggests that, in particular, its C-terminal domain exhibits conformational mobility on a sub-millisecond time scale and thus should readily undergo any

required structural rearrangements upon binding Myo2p. Interestingly, in contrast to the light chains from chicken smooth muscle myosin that are largely insoluble and tend to aggregate in the absence of myosin,⁴ Cdc4p is very soluble and remains monomeric at relatively high concentrations. This may reflect different functional requirements for the ELC in cytokinesis and in muscle contraction. Regardless, the structure of Cdc4p represents the first example of an isolated ELC and, in combination with that the scallop ELC in a crystalline ternary complex, provides a view of the free and bound conformations of the essential myosin light chain.

In the absence of direct structural information on Cdc4p bound to its target sequence(s) in Myo2p, the scallop ELC-RLC-myosin head piece complex reveals possible explanations for the conditionally lethal phenotypes resulting from several point mutations in this *S. pombe* protein. For example, the temperature sensitivity of Cdc4p-G82D may arise from disruption of the expected shallow hydrophobic pocket that is predicted to be generated in the bound semi-open conformation. Although this residue is not well conserved among ELCs, it always contains a nonpolar side chain. As indicated by the NMR characterization of Cdc4p-G82D, an aspartic acid residue at position 82 may result in a disruption of the packing between helices E and F and thus lead to a subtle alteration in the overall structure of the C-terminal domain. As the temperature is increased, this could destabilize the binding of Cdc4p to Myo2p and thereby disrupt cytokinesis. In a similar manner, the G107S mutation would alter the docking of the C-terminal domain of Cdc4p onto an IQ motif, as the residues in the linker between the two EF-hands (including position 107) are important for this binding event (42). In contrast, the G19E mutation may not have a direct effect on the binding of Cdc4p to the IQ motif but rather could affect the stabilization of the ternary myosin complex involving Cdc4p in the ELC position and a second protein in the RLC position. This hypothesis derives from the observation that the region in the scallop ELC equivalent to Gly-19 in yeast Cdc4p is important for inter-light chain hydrogen bonding (42) (Fig. 4).

Cdc4p Interacts with Several Protein Partners—The early observation by Nurse *et al.* (12) that conditionally lethal alleles *cdc4-G19E* and *cdc4-G107S* complemented one another under restrictive temperatures in diploid cells has recently been extended to include the combinations of *cdc4-F12L/cdc4-G82D* and *cdc4-F12L/cdc4-R33K* (49). This interallelic complementation cannot be easily rationalized if the only role of Cdc4p is that of an ELC. One possible explanation is that Cdc4p may have two or more essential and independent functions, each being selectively disrupted by a mutation. Consistent with this possibility, Cdc4p appears to bind several proteins besides Myo2p. For example, in *myo2*-null mutants, Cdc4p is still recruited to the contractile ring, perhaps by associating with Myp2p, a second myosin heavy chain found in *S. pombe*. Furthermore, a synthetic lethal genetic interaction between *cdc4* and *rng2*, a gene encoding a contractile ring protein similar to human IQGAP1, has been documented (15). This latter protein is known to bind actin and calmodulin, as well as to regulate Rho GTPases (15). Phenotypic analysis has led to the suggestion that Cdc4p and Rng2p may be involved in organizing actin cables into rings (43).

As presented in the accompanying paper (49), using a yeast two-hybrid screen, we have identified two additional Cdc4p-binding proteins not obviously associated with cytokinesis. The first of these is a putative phosphatidylinositol 4-kinase (PI 4-kinase). This interaction is dependent only on the C-terminal

⁴ L. F. Saltibus, personal communication.

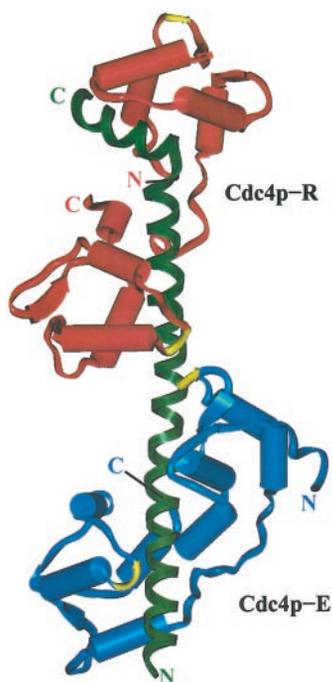


FIG. 4. A proposed model of interallelic complementation in which two Cdc4p proteins bind to the S1 neck region of the myosin heavy chain. The figure illustrates Cdc4p, modeled according to the crystallographically determined structure of the scallop muscle ternary complex (42) as the equivalent of an RLC (red) and ELC (blue), bound to the neck region of the myosin heavy chain (green). Note the different conformations of the ELC and RLC with respect to interhelical angles and that both light chain-heavy chain and light chain-light chain interactions are required to stabilize the complex. The positions of Gly-19 and Gly-107 in Cdc4p are highlighted in yellow. According to this model of interallelic complementation, single point mutations, such as G19E or G107S, disrupt the complex in part by destabilizing necessary interactions between Cdc4p in the two light chain positions, leading to a conditionally lethal phenotype. However, in a diploid cell expressing copies of both mutant alleles, a wild-type interface exists between Cdc4p-G19E in the RLC position and Cdc4p-G107S in the ELC position, thereby allowing formation of a ternary complex as required for cytokinesis.

domain of Cdc4p and is disrupted by the mutation G017S but not F79S or G82D. This implicates residues near position 107 as mediating association with the kinase. Note, however, that viability in *cdc4⁻* strains can be restored by exogenous expression of full-length Cdc4p but not by either its isolated N- or C-terminal domain. Thus, although the interaction of Cdc4p with PI 4-kinase occurs via the C-terminal domain alone, the attached N-terminal domain may be required for biological function, perhaps by mediating the formation of higher order protein assemblies. The second Cdc4p-interacting partner identified by two-hybrid screening is similar to *S. cerevisiae* Vps27p, a protein implicated in vacuolar and endocytic membrane traffic. In contrast to the case of the PI 4-kinase, this interaction is dependent on both domains of Cdc4p and is selectively disrupted by the substitution of F12L. Position 12 is located within the hydrophobic core of Cdc4p, and thus this mutation may perturb, albeit subtly, the structure of its entire N-terminal domain. Possible biological implications of these two partnerships are discussed in the accompanying paper (49). However, it remains to be established which, if any, of these interactions represent essential functions of Cdc4p in *S. pombe* and thus could account for the observed phenomenon of interallelic complementation.

A Structural Model How Cdc4p May Interact with Myosin as Both an ELC and RLC—A second mechanism by which interallelic complementation may arise is through the assembly of

mutant forms of a protein into a functional complex in diploid cells. In Fig. 4, we propose a model in which two Cdc4p molecules could bind to the neck region of each Myo2p heavy chain, in a fashion structurally equivalent to the ternary complex involving an ELC, RLC, and the headpiece of scallop muscle myosin heavy chain (37, 41, 42). The main features of the model are as follows: (i) one Cdc4p interacts with Myo2p as the structural equivalent to an ELC bound to the myosin heavy chain; (ii) a second Cdc4p interacts with Myo2p as the structural equivalent of an RLC bound to muscle myosin; (iii) the N-domain of Cdc4p (in an ELC-like position) interacts with the C-domain of Cdc4p (in an RLC-like position); (iv) both Cdc4p-Cdc4p and Cdc4p-Myo2p interactions are essential for function.

The key feature of this model is that Cdc4p associates with Myo2p at both the ELC- and RLC-binding sites of the myosin heavy chain. That Cdc4p binds to the first IQ motif of Myo2p was shown in Ref. 13 as mutation of Arg-770 within this motif abolished interactions in coimmunoprecipitation assays. In conventional myosins, the first of two IQ motifs is a binding site for an ELC. Our model suggests that a second Cdc4p could also occupy the RLC-binding site in Myo2p. This hypothesis is based on the following: (i) the NMR-derived structure of Cdc4p, combined with dynamic measurements, indicating that this protein exhibits flexibility and thus could conceivably undergo changes in conformation to resemble those of either the bound ELC or RLC (Table II); (ii) NMR and CD spectroscopic studies of three mutant forms of Cdc4p which reveal that the temperature-sensitive defects in cytokinesis are caused by disruption of function rather than of overall structure; (iii) interallelic complementation that is readily explained if a ternary complex comprising two Cdc4p is formed with myosin; (iv) the demonstration that the two domains of Cdc4p are not functionally independent as only the intact full-length protein can rescue mutant phenotypes (49); and (v) analogy to the crystallographic structure of the scallop RLC-ELC-myosin ternary complex, in which important interactions exist not only between each light chain and myosin but also between the C-domain of the ELC and the N-domain of the RLC (42).

It should be noted that three exhaustive genetic screens for *cdc⁻* mutants failed to identify another protein that could serve as an RLC (12, 43, 44). In *Dictyostelium*, mutations in either its ELC or RLC cause cytokinesis defects and loss of viability (45–48), suggesting that such a protein should have been detected if essential for cell division. However, a gene that presumably encodes a small EF-hand protein was recently annotated as an RLC based on sequence similarity (33.9% identity in 171 amino acids) to *Drosophila melanogaster* myosin RLC (Sanger Center *S. pombe* sequencing group, accession number CAB54151). This RLC-like protein also localizes to the contractile ring and appears to interact with Myo2p at the second IQ motif, a recognized binding site for RLC in conventional myosins.² Since the gene encoding this protein was not detected as a *cdc⁻* mutant, it remains to be established if it plays an essential role in cytokinesis. It is also possible that Cdc4p and the RLC-like protein may bind to myosin interchangeably or at different times during the formation or function of the contractile ring, allowing for the formation of the postulated Cdc4p-Cdc4p-Myo2p complex.

In the model of Fig. 4, both domains of Cdc4p are required to associate with Myo2p. By analogy to the scallop RLC-ELC-myosin ternary complex, the binding of Cdc4p in the RLC position is required to stabilize the weak binding of the Cdc4p N-domain in the ELC position. This is consistent with the experimental observations that the two domains of Cdc4p are not functionally independent and that expression of intact

Cdc4p is required to rescue the cytokinesis defects observed in yeast with mutant alleles of Cdc4p. For example, expression of full-length wild-type Cdc4p in cells bearing mutations in the N-domain (G19E) or C-domain (G107S) restored viability at the restrictive temperature, whereas expression of the N-domain or C-domain alone or in combination did not (49). These results are similar to those observed in *Dictyostelium* where expression of an ELC with deletions in either the N terminus or C terminus abolished binding to myosin and failed to rescue cytokinesis defect of ELC^{null} cells (47). The fact that free Cdc4p is monomeric in solution indicates that, as with muscle ELC and RLC, the postulated interactions are weak, occurring only when both light chains are bound to the myosin heavy chain.

Within the context of the proposed model, the structure of Cdc4p provides a rationale to explain how single point mutations cause temperature-dependent failure of cytokinesis. A key feature of the model is that two Cdc4p interacts in different ways with the heavy chain of Myo2p depending upon their position as the structural equivalents to an ELC and RLC. Thus, a single point mutation may have a destabilizing effect when Cdc4p is in one position but not in the other, providing a rationale to explain the observed cases of interallelic complementation. For instance, position 107 occurs in a highly conserved region of Cdc4p and the glycine to serine substitution causes subtle structural perturbations throughout its C-terminal domain. These perturbations may interfere with potential hydrogen bonding between the glutamine side chain in position 2 of the second heavy chain IQ motif and the carbonyls of Leu-107 and Glu-108 of Cdc4p, thereby weakening the binding of Cdc4p-G107S to Myo2p. Furthermore, in the scallop muscle RLC-ELC-myosin ternary complex, the equivalent residues to Gly-107 of Cdc4p in the RLC position (Fig. 4, red) hydrogen-bonds via its backbone amide and carbonyl atoms to the backbone amide and carbonyls of the equivalent residues to Arg-17 and Gly-21 of Cdc4p in the ELC position (Fig. 4, blue). Strikingly, these latter residues surround the G19E mutation in the N-domain of Cdc4p. Thus, in addition to disrupting direct interactions with Myo2p, mutation of either residue 19 or 107 may destabilize the association of two Cdc4p molecules acting as the structural equivalents of the ELC and RLC. Since interaction between the two light chains is required for ternary complex formation, the mutations would lead to a conditionally lethal phenotype. Extending this argument, the fact that diploid cells expressing G107S and G19E mutant alleles are viable at the restrictive temperature can be explained with G107S mutant protein in the ELC position and G19E in the RLC position. This would preserve an intact wild-type interface between the two Cdc4p (Fig. 4). Similar arguments can be made regarding the complementation of Cdc4p-F12L and Cdc4p-G82D, with amino acid substitutions in their N-terminal and C-terminal domains, respectively. In the case of Cdc4p-F12L and Cdc4p-R33K, with mutations in the same domain, a wild-type complex with Myo2p may arise if one amino acid substitution disrupts the binding in the conformation required of an ELC but not an RLC, and vice versa for the second substitution. This situation is more akin to the case in which interallelic complementation arises when a protein has two independent functions, namely that of an ELC and RLC.

Functional Considerations—Location- and time-dependent assembly and activity of a contractile ring are primary features of cytokinesis (1, 2, 12). The function of Cdc4p as a light chain has been largely inferred by analogy to known functions of myosin light chains in muscles. The muscle analogy may not be entirely appropriate. The requirements in muscle cells for variable speed and force development are unlikely features of cytokinesis. Conversely, the contractile ring is a transient struc-

ture, assembled and disassembled in minutes. We suggest that Cdc4p has the potential to interact with Myo2p in the equivalent mode of both the ELC and RLC. Does it follow from the muscle analogy that as an RLC, metal binding or phosphorylation of Cdc4p controls the activity of the contractile ring? There is currently no evidence for this. As shown in this study, Cdc4p does not bind calcium. Furthermore, fission yeast expressing mutated versions of Cdc4p that cannot be phosphorylated at serines 2 or 6 (Fig. 1) grow and divide normally (40). Could Cdc4p be involved in regulating the assembly or disassembly of the contractile ring? Detection of Cdc4p in improperly formed medial rings in Myo2p-deficient cells (13), as well as potential interactions with Rng2p, suggest roles beyond that of simply an ELC. Finally, what are the functional consequences of the recently detected partnerships of Cdc4p with a putative PI 4-kinase and a Vps27p-like protein (49). The structure of Cdc4p and the proposed models for interallelic complementation provide several clear and testable predictions for addressing these questions and thereby delineating the role of this protein in the assembly and function of the contractile ring in fission yeast.

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