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Localization of Fission Yeast Type II Myosin, Myo2, to the Cytokinetic Actin Ring Is Regulated by Phosphorylation of a C-Terminal Coiled-Coil Domain and Requires a Functional Septation Initiation Network

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Myo2 truncations fused to green fluorescent protein (GFP) defined a C-terminal domain essential for the localization of Myo2 to the cytokinetic actin ring (CAR). The localization domain contained two predicted phosphorylation sites. Mutation of serine 1518 to alanine ($S^{1518}A$) abolished Myo2 localization, whereas Myo2 with a glutamic acid at this position ($S^{1518}E$) localized to the CAR. GFP-Myo2 formed rings in the septation initiation kinase (SIN) mutant *cdc7-24* at 25°C but not at 36°C. GFP-Myo2$S^{1518}E$ rings persisted at 36°C in *cdc7-24* but not in another SIN kinase mutant, *sid2-250*. To further examine the relationship between Myo2 and the SIN pathway, the chromosomal copy of *myo2*$^+$ was fused to GFP (strain *myo2-gc*). Myo2 ring formation was abolished in the double mutants *myo2-gc cdc7.24* and *myo2-gc sid2-250* at the restrictive temperature. In contrast, activation of the SIN pathway in the double mutant *myo2-gc cdc16-116* resulted in the formation of Myo2 rings which subsequently collapsed at 36°C. We conclude that the SIN pathway that controls septation in fission yeast also regulates Myo2 ring formation and contraction. Cdc7 and Sid2 are involved in ring formation, in the case of Cdc7 by phosphorylation of a single serine residue in the Myo2 tail. Other kinases and/or phosphatases may control ring contraction.

INTRODUCTION

The critical events for the successful execution of cytokinesis are the recruitment of the proteins that form the contractile ring to the incipient cleavage site and the timing of ring contraction such that the newly segregated sister chromatids are equally partitioned into two daughter cells, each of which contains an allocation of the parental cell cytoplasm and organelles. In cells possessing a cell wall, such as the fission yeast, *Schizosaccharomyces pombe*, the timing and positioning of the contractile machinery on the inside of the cell membrane must be additionally coordinated with the formation of a new cell wall structure, the septum, on the outside of the cell. The analysis of *S. pombe* mutants defective in septum timing and placement have provided important clues as to the nature of these control mechanisms (Le Goff *et al.*, 1999). Fission yeast cells divide by means of a contractile actin ring (CAR) that both positions the cytokinetic septum and determines its structural and functional integrity (Marks and Hyams, 1985). The CAR contains two type II myosins encoded by the genes *myo2+* (Kitayama *et al.*, 1997; May *et al.*, 1997) and *myp2+/myo3+* (Bezanilla *et al.*, 1997; Motegi *et al.*, 1997). Myo2 is an essential component of the CAR (Kitayama *et al.*, 1997; May *et al.*, 1997). Cytokinesis can proceed in the absence of Myp2 but less efficiently than when it is present (Bezanilla *et al.*, 1997, Motegi *et al.*, 1997, 2000; Mulvihill *et al.*, 2000). Bezanilla *et al.* (2000) have claimed Myo2 assembles into the CAR in advance of Myp2, whereas Motegi *et al.* (2000) conclude that the two myosins arrive at the division site coincidentally. Myo2 dimerizes like other myosin IIs but Myp2 appears to be monomeric (Bezanilla and Pollard, 2000). The two type 2 myosins are associated with the same light chains, Cdc4 (McCollum *et al.*, 1995; Naqvi *et al.*, 1999; Motegi *et al.*, 2000) and Rlc1 (Le Goff *et al.*, 2000; Naqvi *et al.*, 2000).

We have proposed previously that recruitment of Myo2 to the CAR is regulated by the septation initiation network (SIN), leading to septum formation and cytokinesis (Mulvihill *et al.*, 2000). At the heart of this signal transduction pathway are three protein kinases, Cdc7, Sid1, and Sid2 (Fankhauser and Simanis, 1994; Sparks *et al.*, 1999; Guertin *et al.*, 2000) and their activating GTPase Spg1 (Schmidt *et al.*, 1997). The timing of Spg1 activation is determined by a two-component GTPase-activating protein consisting of Cdc16 and Byr4 (Furge *et al.*, 1998). All of these components, with the exception of Sid1, have homologs in the budding yeast mitotic exit network (Hoyt, 2000). Mutations in Cdc7,

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Table 1. Strains used in this study

Strain	Genotype	Source
JS229	h$^-$ *cdc7-24 leu1-32*	Nurse *et al.*, 1978
JS380	h$^-$ *cdc4-8 ade6-210 ura4-d18 myo2-gc*	This study
JS460	h$^-$ *myo2-E1 ade6-210 ura4-d18 his3-d*	Balasubramanian *et al.*, 1998
JS476	h$^-$ *ade6-210 leu1-32 ura4-d18 myo2-gc*	This study
JS646	h$^-$ *cdc7-24 ade6-210 leu1-32 myo2-gc*	This study
JS647	h$^-$ *cdc11-136 ade6-210 leu1-32 myo2-gc*	This study
JS766	h$^-$ *rng2-d5 ade6-210 leu1-32 ura4.d18 myo2-gc*	This study
JS667	h$^-$ *cdc7-A20 leu1-32*	Sohrmann *et al.*, 1998
JS680	h$^-$ *cdc16-116 ade6-210 leu1-32 myo2-gc*	This study
JS720	h$^-$ *ade6-210 leu1-32 ura4-d18*	Grimm *et al.*, 1988
JS741	h$^+$ *ade6-210 leu1-32 ura4-d18 his2-myo2-gc*	This study
JS748	h$^-$ *sid2-250 ade6-216 leu1-32 ura4.d18*	Balasubramanian *et al.*, 1998
JS755	h$^-$ *sid2-250 ade6-210 leu1-32 ura4.d18 myo2-gc*	This study

Sid1, Sid2, and Spg1 abolish septation, whereas mutations in Cdc16 and Byr4 drive cells into cytokinesis and they become multiseptate (Minet *et al.*, 1979; Fankhauser et al., 1993). A similar phenotype is seen when Cdc7 and Spg1 are overexproduced (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997). The evidence that the SIN pathway also regulates Myo2 function includes a strong genetic interaction between a *myo2* deletion strain and *cdc7-24* (May *et al.*, 1997) and the reduced efficiency of septation in *myo2* mutants after overexpression of Spg1 or Cdc7, or the inactivation of Cdc16 (Mulvihill *et al.*, 2000). This raises the question as to whether the Myo2 heavy chain is a substrate for Cdc7 or a downstream kinase, or whether regulation could work through an associated light chain. Cdc4 function does not appear to be regulated by phosphorylation (although it is a phosphoprotein; McCollum *et al.*, 1999) and both Cdc4 and Rlc1 lack the serine at position 19 whose phosphorylation is associated the activation of myosin II ATPase activity by myosin II regulatory light chain in nonmuscle cells. We have therefore examined the role of Myo2 heavy chain phosphorylation in the recruitment of this myosin to the CAR.

MATERIALS AND METHODS

Cell Culture and Strains

Cell culture and maintenance were carried out according to Moreno *et al.* (1991). Experiments were carried out in Edinburgh minimal medium 2 (EMM2). Repression of the *nmt1* promoter (Maundrell 1993) was achieved by the addition of 4 μM thiamine to the growth medium. To examine the effect of overexpressing the *myo2*$^+$ constructs, cells were grown for 24 h to mid-log phase in EMM2 supplemented with thiamine to repress transcription. Cells were then washed three times in EMM2 lacking thiamine and resuspended to a suitable cell density (2×10^6 cells/ml), and cultured overnight at 25°C. Phenotypes were examined after 22 h of fusion protein expression. The strain *myo2-gc* was created with the use of the method of Bahler *et al.* (1998b) Genetic crosses to create double mutants with mutants in the SIN pathway were carried as described in Egel *et al.* (1994). The strains used in this article are listed in Table 1.

Molecular Genetic Manipulations

The amino terminal half (the first 2302 base pairs) of the *myo2*$^+$ gene was isolated from the plasmid pBlue*myo2*$^+$ (May *et al.*, 1997) as an *Sal*I-*Bam*HI fragment and was ligated into *Sal*I-*Bam*HI cut pREP41N*egfp*$^+$ (Craven *et al.* 1998) to give the plasmid pREFP41*gfp-myo2*^{768}. The carboxyl-terminal half (the last 2275 base pairs) of the *myo2*$^+$ gene was isolated from pBlue*myo2*$^+$ as a *Bam*HI-*Bam*HI fragment and was ligated into the *Bam*HI site of pREP41*gfp-myo2*^{768}, to give pREP41*gfp-myo2*$^+$. The C-terminal 3552 base pairs of *myo2*+ were isolated as an *Nde*I-*Sma*I fragment from pREP41*gfp-myo2*$^+$ and ligated into pREP41N*egfp*$^+$ to create p41*gfp-myo2*$^{343–1526}$. A *Sal*I-*Sca*I fragment containing a truncated form of *myo2*$^+$ was purified from pBlue*myo2*$^+$ and ligated into pREP41N*egfp*$^+$, to create p41*gfp-myo2*^{819}. A *Sal*I-*Bgl2* fragment was purified from pBlue*myo2*+, and ligated into pREP41*gfp*$^+$, to create p41GFP-*myo2*^{1228}. A *Sal*I-*Sna*B1 myo2$^+$ fragment was eluted from pBlue*myo2*$^+$ and ligated into pREP41*gfp*$^+$ to create p41*gfp-myo2*^{1336}. A *Bgl2-Bam*HI fragment, which contains the sequence encoding the final 303 amino acids of Myo2 was purified from pBlue*myo2*$^+$ and ligated into pREP41*gfp*$^+$ to create p41*gfp-myo2*$^{1228–1526}$. p41GFP-*myo2*$^{1228–1526}$ was digested with the use of *Sna*B1 and *Sma*I, and the subsequent plasmid was religated together to create p41*gfp-myo2*$^{1228–1335}$. A *Bgl2-Msc1* fragment was purified from p41*gfp-myo2*$^+$ and ligated into pREP41*gfp*$^+$, which had been cut with *Nde*I and *Sma*I to create p41*gfp-myo2*$^{1228–1448}$.

Site-directed mutagenesis was carried out with the use of the site-directed mutagenesis kit from Stratagene (La Jolla, CA) with pREP41N*egfp-myo2*$^+$ as a template and the primers listed in Table 2.

Table 2. Oligonucleotides used in this study

Oligonucleotide name	Sequence (5′ → 3′)
S1505A forward	GTGAACAAAGATGCGTTAATAGATGTG
S1505A reverse	CACATCTATTAACGCATCTTTGTTCAC
S1518A forward	GATCGCATTGCAGCCCTTGAAGATGAG
S1518A reverse	CTCATCTTCAAGGGCTGCAATGCGATC
S1518E forward	GATCGCATTGCAGAACTTGAAGATGAG
S1518E reverse	CTCATCTTCAAGTTCTGCAATGCGATC
myo2-gc forward	AAGCCATTCATTATTGCAAATTAAGAA-TCAAGCAATGTACTGGCCGTATTAT-ATATACATCATAATGAAAGCTCAGA-ATTCGAGCTCGTTTAAAC
myo2-gc reverse	ATGGAGAGATTTATACATATATTGGAC-ATGTGTTGATTTCTGTAAATCCATTT-CGAGACCTGGGTATTTATACGCGGA-TCCCCGGGTTAATTAA

The resultant plasmids were sequenced to confirm that only the predicted mutagenesis had taken place.

Localization

For GFP autofluorescence microscopy cells were fixed in 3.7% formalin for 10 min, washed once in phosphate-buffered saline containing 0.1% Triton X-100, and twice in phosphate-buffered saline. Cells were mounted onto slides and DNA stained with the use of 4,6-diamidino-2-phenylindole as described in Moreno *et al.* (1991). Images obtained with Zeiss Axiophot microscope fitted with a 1.4 numerical aperture 64× objective were captured with the use of OpenLab computer software (Improvision, Coventry, United Kingdom).

RESULTS

The ability of a series of GFP-tagged truncations of *myo2*$^+$ to localize to the CAR was examined by fluorescence microscopy. Empty vector and full-length, N-terminally GFP-tagged Myo2 (GFP-Myo2) served as negative and positive controls, respectively (Figure 1, ai–aiv and bi–biv). The GFP-Myo2 construct was fully functional based on 1) its localization to the CAR (Figure 1, bi) and subsequent contraction and 2) its ability to rescue the mutants *myo2-E1* and *myo2*Δ (our unpublished data). The leaky nature of the thiamine-repressible *nmt41* promoter allowed us to compare each construct at low (+thiamine) and high (−thiamine) levels of expression. At low levels of expression, two of the constructs, GFP-Myo2$^{343–1526}$ (lacking the region of the head containing the ATP-binding site) and GFP-Myo2$^{1228–1526}$ (encoding the carboxyl terminal half of the tail), located to the CAR at comparable efficiency to the full-length protein (Figure 1, bi, ci, and di). At higher levels of expression, both constructs were toxic and GFP-Myo2 accumulated as aggregates at the cell equator, typical of Myo2 overexpression (Figure 1, ciii and diii; cf., the full-length protein in biii). The construct GFP-Myo2$^{1228–1448}$ (the carboxyl-terminal half tail lacking the final 77 amino acids) failed to localize to the CAR (our unpublished data). In fact, no construct lacking this region localized to the CAR (GFP-Myo2$^{1–768}$; Figure 1, ei; GFP-Myo2$^{1–819}$, Figure 1 fi; GFP-Myo2$^{1–1228}$, Figure 1 gi; GFP-Myo2$^{1–1335}$, our unpublished data). At higher levels of expression, constructs lacking the tail, e.g., GFP-Myo2$^{1–768}$ (Figure 1, eiii), GFP-Myo2$^{1–819}$ (Figure 1, fiii) or just the C-terminal 77 amino acids, GFP-Myo2$^{1–1228}$ (Figure 1, giii) and GFP-Myo2$^{1–1335}$ (our unpublished data), localized to actin structures at the cell tips as well as the equator. Thus, the carboxyl-terminal region of the Myo2 tail not only determines the localization of Myo2 to the CAR at cytokinesis but also prevents Myo2 from interacting indiscriminately with actin at other points of the cell cycle. We therefore refer to this region as the localization domain (LD). However, expression of the LD alone, either in the presence or absence of thiamine, did not result in the appearance of GFP rings, neither was its overexpression toxic (our unpublished data). Thus, the LD is essential but not sufficient for the localization the Myo2 to the CAR. These results are summarized in Figure 2. Because the contribution of the second fission yeast myosin II gene, *myp2*$^+$, to cytokinesis remains to be precisely defined, we expressed all of the above-mentioned constructs in *myp2*Δ (Mulvihill *et al.*, 2000) in both the presence and absence of thiamine. In no case did we detect a change in Myo2 localization.

Because Myo2 is known to be a phosphoprotein (McCollum *et al.*, 1999), an obvious way in which the localization domain might regulate Myo2 recruitment to the CAR is by phosphorylation. The Myo2$^{1448–1526}$ (LD) sequence was therefore examined for potential phosphorylation consensus sites with the use of the NetPhos 2.0 Protein Phosphorylation Prediction Server (http://www.cbs.dtu.dk/services/NetPhos/). Two peptides were found that had a high probability (>0.98) of being consensus sites for known serine-threonine protein kinases (serines at residues 1505 and 1518; Figure 3A). To investigate whether phosphorylation of these sites was required for the protein localization, the serine residues were individually replaced with alanines by site-directed mutagenesis of the plasmid pREP41N*gfp-myo2*$^+$. Mutation of serine 1505 (Myo2S^{1505}A) resulted in the formation of less intense GFP-Myo2 rings (Figure 3B, b, arrows) and spots of GFP fluorescence throughout the cell. In contrast, replacing the serine at residue 1518 with alanine (Myo2S^{1518}A) abolished GFP-Myo2 ring formation, either in the presence or absence of thiamine (Figure 3B, c). This mutation also abolished the typical Myo2 overexpression phenotype (May *et al.*, 1997; Bezanilla and Pollard, 2000). To examine whether changing serine 1518 to a negatively charged residue such as glutamic acid that would mimic phosphorylation at this position, the pREP41*gfp-myo2*$^+$ plasmid was again mutated, and the resultant plasmid, pREP41*gfp-myo2S*^{1518}*E*, introduced into yeast cells. Myo2S^{1518}E was incorporated into the CAR, which contracted with wild-type efficiency (Figure 3B, d and C). Overproduction of this mutant was less toxic than the wild-type protein.

We suggested previously that the incorporation of Myo2 into the CAR was under the regulation of the SIN pathway that controls septum formation in fission yeast (Mulvihill *et al.*, 2000). To investigate this further we expressed GFP-Myo2 in the mutants *cdc7-24 and cdc7-A20*. At 25°C GFP-Myo2 was incorporated into the CAR. When the culture was transferred to 36°C, to fluorescent Myo2 rings disappeared within 3 h (Figure 4, A and B) but returned within 2 h when cells were restored to 25°C (our unpublished data). Thus, Cdc7 function is required to maintain the integrity of the CAR. We repeated this experiment with Myo2S^{1518}E. In this case, Myo2 rings persisted at 36°C although they did not contract (Figure 4D). These data point to serine 1518 being a target, either directly or indirectly, of Cdc7. To resolve this point, we expressed Myo2S^{1518}E in another SIN kinase mutant, *sid2-250*. As in *cdc7-24*, both GFP-Myo2 and Myo2S^{1518}E formed rings at 25°C; neither, however, persisted at the restrictive temperature (Figure 4C).

To further investigate the relationship of Myo2 to the SIN pathway we created the strain *myo2-gc* in which the chromosomal copy of *myo2*$^+$ was fused to the gene encoding GFP to produce a C-terminally tagged Myo2 under the control of its own promoter. *myo2-gc* had wild-type morphology and showed normal growth characteristic at all temperatures tested. We created double mutants with *myo2-gc* and mutants in all of the known components in the SIN pathway and some of the genetic interactions are summarized in Figure 5. *myo2-gc* showed synthetic lethality with *cdc7.24* at the semipermissive temperature (29°C; Figure 6a). The interaction with *sid2-250* was stronger and *myo2-gc sid2-250* showed poor growth even at the permissive tempera-

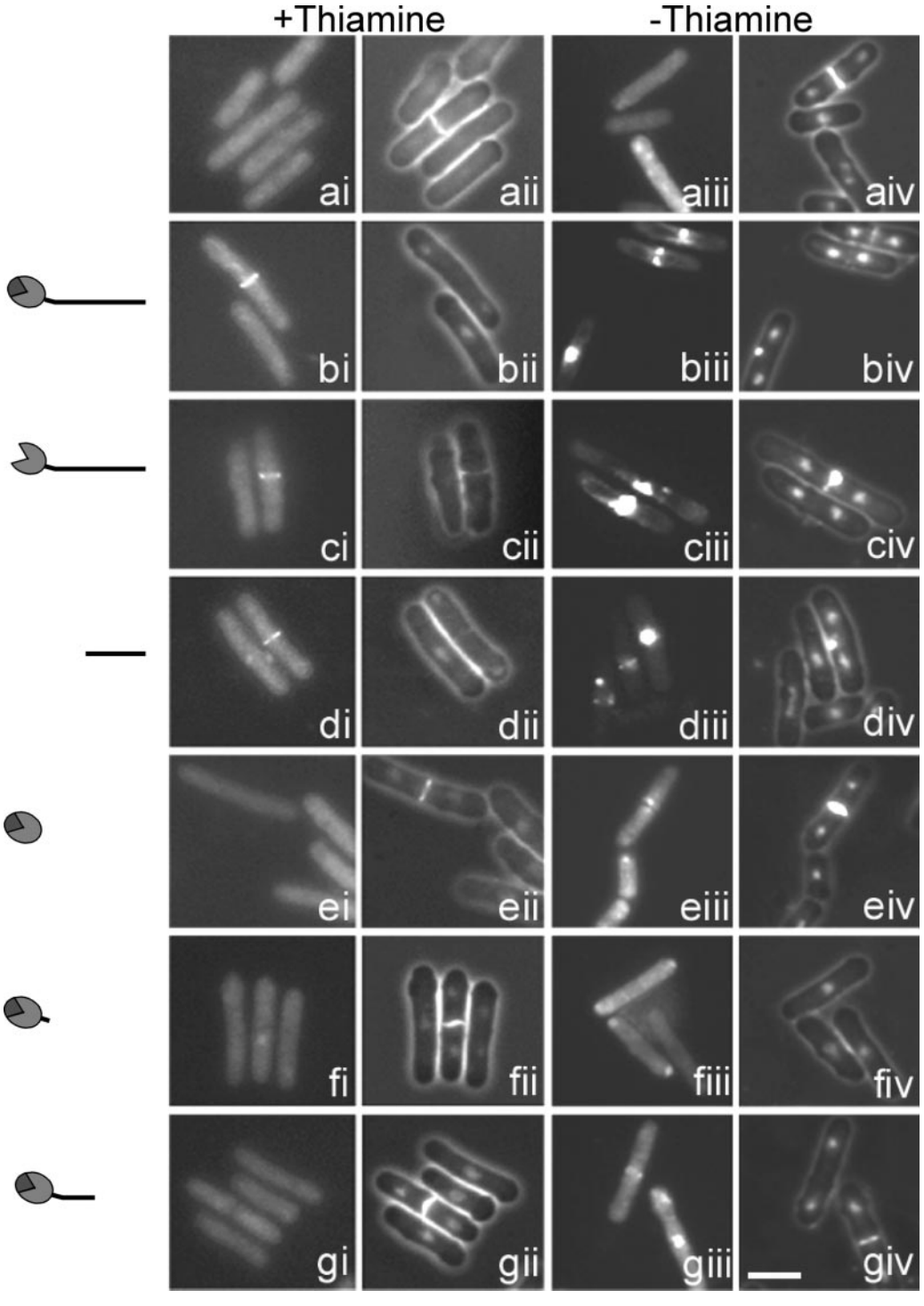


Figure 1. Localization of GFP-Myo2 truncations. Wild-type cells were transformed with plasmids that contained DNA encoding GFP (a), N-terminally GFP-tagged full-length Myo2 (b), and GFP-tagged Myo2 truncations (c–g). The truncations are shown in cartoon form down the left-hand side of the figure. (i) GFP fluorescence. (ii) 4,6-Diamidino-2-phenylindole/Calcofluor staining under repressed conditions (+ thiamine). (iii and iv) As in i and ii but under derepressed conditions (18 h in medium lacking thiamine). GFP alone showed diffuse cytoplasmic fluorescence (ai and aiii). Full-length GFP-Myo2 localized to the CAR in repressed conditions (bi) and accumulated as aggregates at the cell equator when the construct was overexpressed (biii). Similar results were obtained in constructs Myo2$^{343–1526}$ and Myo2$^{1228–1526}$ containing the C-terminal half of the tail (amino acids 1228–1526) (c and d). In contrast, GFP-Myo2 truncation fusions lacking this C-terminal domain (Myo2$^{1–768}$, Myo2$^{1–819}$, and Myo2$^{1–1228}$) showed no discrete localization in repression conditions (ei, fi, and gi). When overexpressed, these fusions localized indiscriminately to actin structures (eii, fii, and gii). Bar, 10 μm.

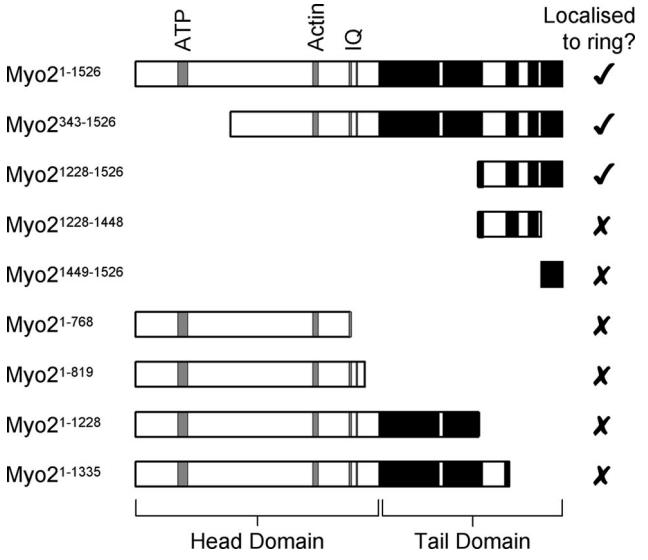


Figure 2. Diagram summarizing which constructs localized to the CAR and which did not. Myo2$^{1-1526}$ represents the full-length Myo2 protein. The ATP and actin binding domains are shown as shaded boxes and the two IQ motifs (May *et al.*, 1997) as vertical lines. The tail domain consists of segments of predicted coiled coil (black boxes) interrupted by proline residues. The final 298 amino acids of the Myo2 protein are required and sufficient for the recruitment of Myo2 to the CAR. Removing the final 77 amino acids abolished Myo2 localization to the CAR.

ture (Figure 6b). Despite repeated attempts, we were unable to create the double mutant *myo2-gc sid1-25.* We next examined Myo2 ring formation in *myo2-gc cdc7-24* and *myo2-gc sid2-250.* In both cases, rings reversibly disappeared at the restrictive temperature, as they had when Myo2 was expressed from a multicopy plasmid. In some cells a dot of fluorescence was observed at the cell tips (Figure 6, c and d). Because the SIN pathway appears to be essential for CAR formation we examined the effect of uncontrolled SIN on Myo2 incorporation into the CAR. The temperature-sensitive mutant *cdc16-116* undergoes multiple rounds of septation at the restrictive temperature. The double mutant *myo2-gc cdc16-116* showed synthetic lethality at 31°C (Figure 7A). When these cells were raised to the nonpermissive temperature for 3 h, Myo2 rings collapsed (Figure 7, B–E) and 80% of cells accumulated a single bright dot at the cell equator (Figure 7, C and E). Whereas control *cdc16-116* cells in a *myo2*$^{+}$ background formed multiple septa (Figure 7D, inset), *myo2-gc cdc16-116* cells rarely formed more than a single, often distorted septum (Figure 7D).

DISCUSSION

Unlike most conventional myosins, the tails of the two yeast myosin IIs consist of short stretches of alpha helix interrupted by proline residues, rather than a continuous extended structure (Bezanilla *et al.*, 1997; May *et al.*, 1998). Nevertheless, the Myo2 tail is involved in the formation of higher order structures (Naqvi *et al.*, 1999; Bezanilla and Pollard, 2000). As with other myosin IIs (Zang and Spudich,

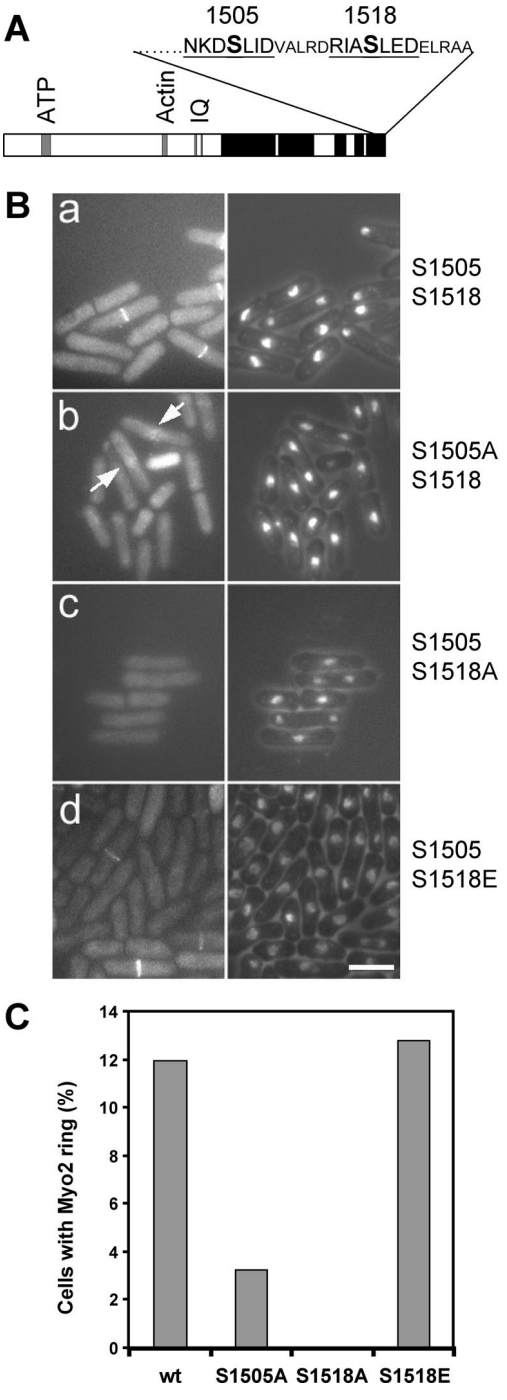
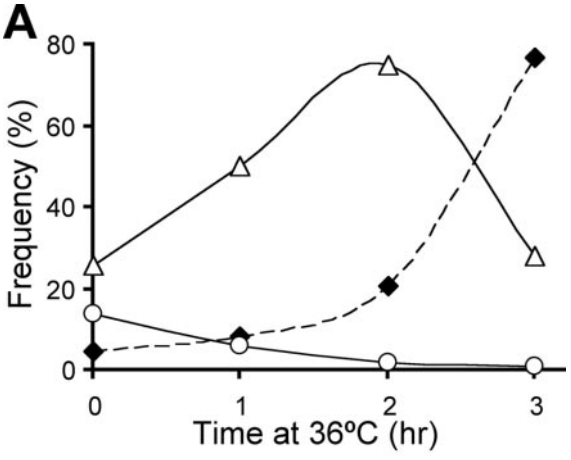


Figure 3. Charge state of two potentially phosphorylated amino acids is intimately associated the recruitment of Myo2 to the actin ring. Mutation of two serines at residues 1505 and 1518 (A) to alanine either inhibits (in the case of S^{1505}A [Bb]) or abolishes (S^{1518}A [Bc]) the recruitment of Myo2 to the actin ring. S^{1505}A rings form but lack the structural integrity of the wild-type protein (B, b, arrows). Mutating serine 1518 to glutamic acid (S^{1518}E) had no significant effect on Myo2 localization nor on the dynamics of CAR contraction (Bd). Quantification of ring formation in the different constructs is shown in C. Bar, 10 μm.



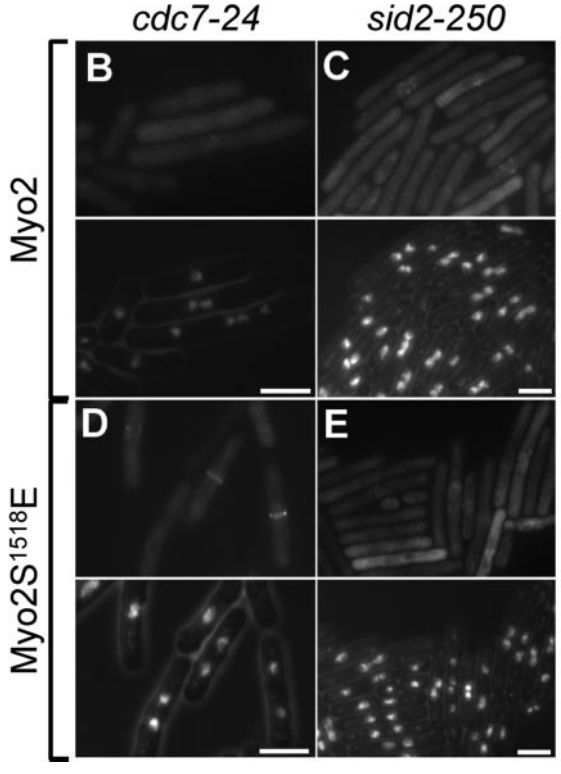


Figure 4. GFP-Myo2 localization to the medial ring is abolished in *cdc7* and *sid2* mutants at the restrictive temperature. (A) *cdc7-24* cells bearing the plasmid *pREPgfp-myo2*$^{+}$ were grown to log phase at 25°C and shifted to 36°C. Samples were taken hourly, fixed, and the proportion of cells with Myo2 rings (○), two nuclei (△), and more than two nuclei (♦) was examined. Note the decline of Myo2 rings in parallel with the expression of the *cdc7* phenotype. Strains bearing mutations in either *cdc7* or *sid2* were transformed with plasmids bearing either *gfp-myo2*$^{+}$ or *gfp-myo2S*^{1518}*E*, grown in the presence of thiamine at the permissive temperature to early-log phase then cultured for 3 h at the restrictive temperature. Cells were fixed to allow GFP-Myo2 and DNA localization to be examined. In both *cdc7-A20* (B) and *sid2-250* (C), the wild-type GFP-Myo2 (S1518) was unable to form rings. In the case of cells expressing GFP-Myo2S^{1518}E, rings persisted in *cdc7-A20*, but not *sid2-250*. Note that the S^{1518}E rings did not contract. Bars, 10 μm.

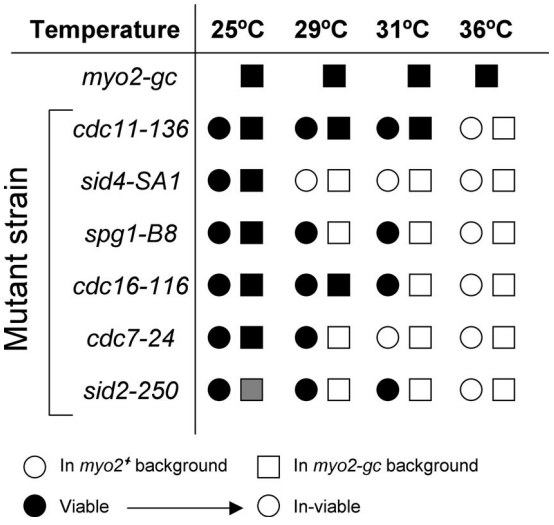


Figure 5. Summary of the genetic interactions between mutations in genes encoding components of the SIN pathway and *myo2-gc*. A strain in which the 3′ terminus of the chromosomal copy of the *myo2*$^{+}$ gene was fused to *gfp*$^{+}$ had similar growth kinetics to wild type at all temperatures and in all media examined. Strains bearing the *myo2-gc* allele in combination with mutants in genes for components of the SIN pathway reduced the permissive temperature for growth in the case of *spg1*, *cdc16*, *cdc7*, and *sid2* but not in the case of *cdc11* and *sid4*.

1998; Yumura and Uyeda, 1997) and, indeed, some unconventional myosins (Reck-Peterson *et al.*, 1999), localization of Myo2 is a function of the tail, neither the head domain (Naqvi *et al.*, 1999), nor the IQ region (Motegi *et al.*, 2000), contributing to the recruitment of Myo2 to the CAR. Here we confirm these previous findings and extend them to show that the C-terminal 77 amino acids of the Myo2 tail, a region with a high probability of forming coiled-coils (Bezanilla *et al.*, 1997; May *et al.*, 1998; Bezanilla and Pollard, 2000), is necessary for Myo2 localization. Constructs lacking this localization domain (Myo2$^{1–768}$, Myo2$^{1–819}$, Myo2$^{1–1228}$, Myo2$^{1–1335}$, Myo2$^{1228–1335}$ and Myo2$^{1228–1448}$) failed to localize to the CAR, whereas constructs containing this region (the full-length protein Myo2$^{1–1526}$, Myo2$^{343–1526}$, and Myo2$^{1228–1526}$) did. Not only does the Myo2 tail domain direct the protein to the CAR but also it prevents it from binding promiscuously to actin at the cell poles. Hence, the specificity of Myo2 recruitment to the CAR resides within a distal tail fragment, which we refer to as the LD. However, the LD alone (Myo2$^{1448–1526}$) failed to localize to the CAR. Thus, the LD is necessary but not sufficient for the recruitment of Myo2 to the CAR and other tail sequences, as yet undefined, appear to contribute to this process. Whether, as in *Dictyostelium*, the localization domain is also an assembly domain remains to be determined but the lack of toxicity of LD overexpression might suggest that it is not.

Myo2 is known to be a phosphoprotein in vivo (McCollum *et al.*, 1999). Examination of the localization domain sequence revealed two serine residues that lie within consensus phosphorylation sites for a number of protein kinases. Mutation of serine 1505 to the nonphosphorylatable amino acid, alanine (S^{1505}A), reduced the efficiency of Myo2

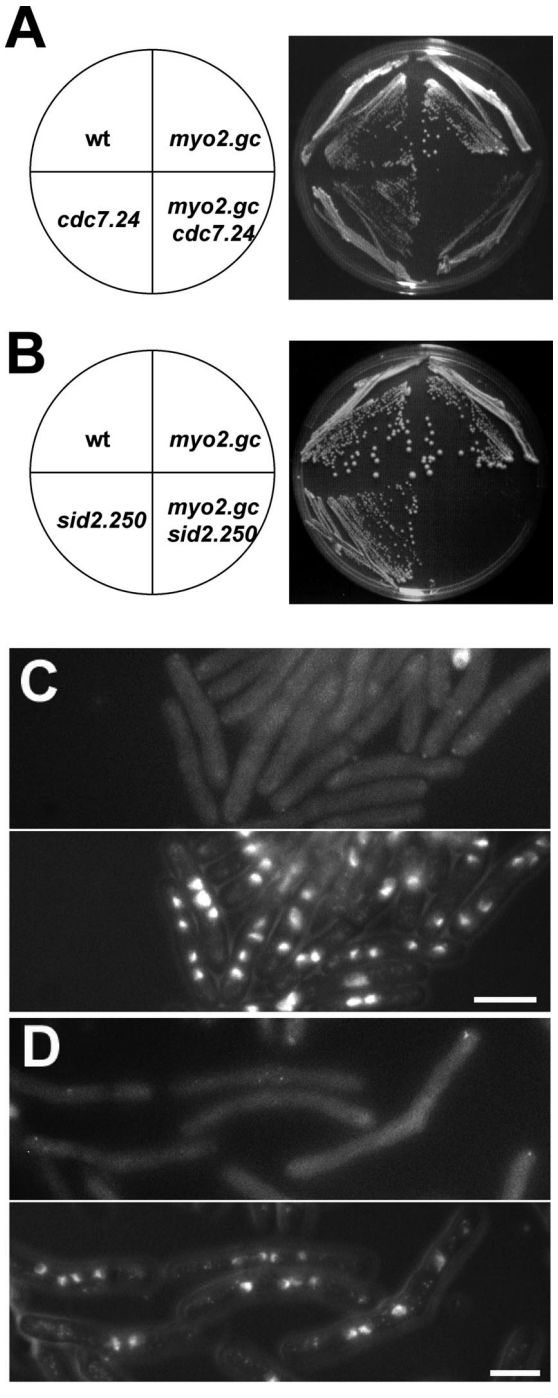


Figure 6. Functional Cdc7 and Sid2 are required to recruit Myo2-GFP to the actin ring. *myo2-gc cdc7-24* (A) and *myo2-gc sid2-250* (B) were unable to grow at 29°C, a temperature at which all three single alleles were viable. When *myo2-gc cdc7-24* cells, which had been grown to early log phase at 25°C were shifted to the nonpermissive temperature Myo2 rings disappeared (C). When log phase *myo2-gc sid2-250* cells were shifted to the nonpermissive temperature of 36°C, Myo2-GFP rings dispersed, but fluorescence remained as dots at the cell tips and cell equator (D). Bars, 10 μm.

localization but the most striking finding was that mutation of serine 1518 to alanine ($S^{1518}A$) abolished both Myo2 localization and the toxicity of Myo2 overexpression. Myo2 carrying the mutation of the same residue to glutamic acid ($S^{1518}E$) to mimic the effect of phosphorylation was recruited to the CAR, which contracted with apparently normal kinetics. This construct also had reduced toxicity when overproduced. These results argue strongly that Myo2 localization is regulated by phosphorylation. They also point to the toxicity of $myo2^+$ overexpression being related to the phosphorylation state of the protein. Double staining of cells overproducing GFP-Myo2 with an actin antibody revealed that nonfunctional protein bound to and sequestered actin at the center of the cell but was unable to form rings (our unpublished data).

The regulation of myosin II function by tail phosphorylation has hitherto only been described in amoebae (reviewed in Brzeska and Korn, 1996). In *Acanthamoeba,* myosin II activity is negatively regulated by phosphorylation of a C-terminal, nonhelical region of its heavy chain (Collins *et al.*, 1982; Ganguly *et al.*, 1992). In *Dictyostelium*, filament assembly and the recruitment of myosin II to the contractile ring require the phosphorylation of three threonine residues at the C terminus (Egelhoff *et al.*, 1991; Lee *et al.*, 1994; Sabry *et al.*, 1997; Shu *et al.*, 1999) by at least two myosin II heavy chain kinases (Ravid and Spudich, 1989, 1992; Kolman *et al.*, 1996). Mutation of all three sites (Egelhoff *et al.*, 1993) or, indeed, just one of them (Nock *et al.* 2000), abolishes myosin function. In fission yeast also, a single charge change abolishes the assembly of Myo2 into the CAR. The recruitment of Myo2 to the CAR must be coordinated with the signal transduction pathway leading to septum formation, the septation initiation network. This includes a number of protein kinases, Plo1 (Okhura *et al.*, 1995; Bahler *et al.*, 1998a; Mulvihill *et al.*, 1999), Cdc7 (Fankhauser and Simanis, 1994), Sid 1 (Guertin *et al.*, 2000), and Sid 2 (Sparks *et al.*, 1999), and their associated regulators (reviewed in Gould and Simanis, 1997; Le Goff *et al.*, 1999; Balasubramanian *et al.*, 2000). Any or all of the above are potential fission yeast myosin II heavy chain kinases. We have shown previously a genetic interaction between *myo2* and *cdc7* mutants (May *et al.*, 1997; Mulvihill *et al.*, 2000) and that the efficiency of septation driven by overexpression of Spg1, the GTPase activator of Cdc7 (Schmidt *et al.*, 1997), or inactivation of Cdc16, one of the components of the Spg1 GTPase-activating protein (Furge *et al.*, 1998), is substantially reduced in the mutant *myo2-E1* (Mulvihill *et al.*, 2000). In this report we show directly that the CAR is dependent for its integrity upon SIN function. Myo2 rings formed with more or less normal efficiency in *cdc7-24* and *cdc7-A20* at the permissive temperature but were abolished at a temperature at which Cdc7 kinase activity is substantially reduced (Fankhauser and Simanis, 1994). Strikingly, the dependency of CAR formation on Cdc7, but not Sid2, was bypassed in $Myo2S^{1518}E$. Thus, both Cdc7 and Sid2 are potential myosin II heavy chain kinases whose functions are required for Myo2 localization to the CAR but only Cdc7 phosphorylates S1518, whereas Sid2 is directed to other residue(s), possibly including S1505. These results clearly require direct biochemical confirmation. We are also aware that although Sid2 has been shown to be associated with the CAR (see below), Cdc7 has not (Sohrmann *et al.*,

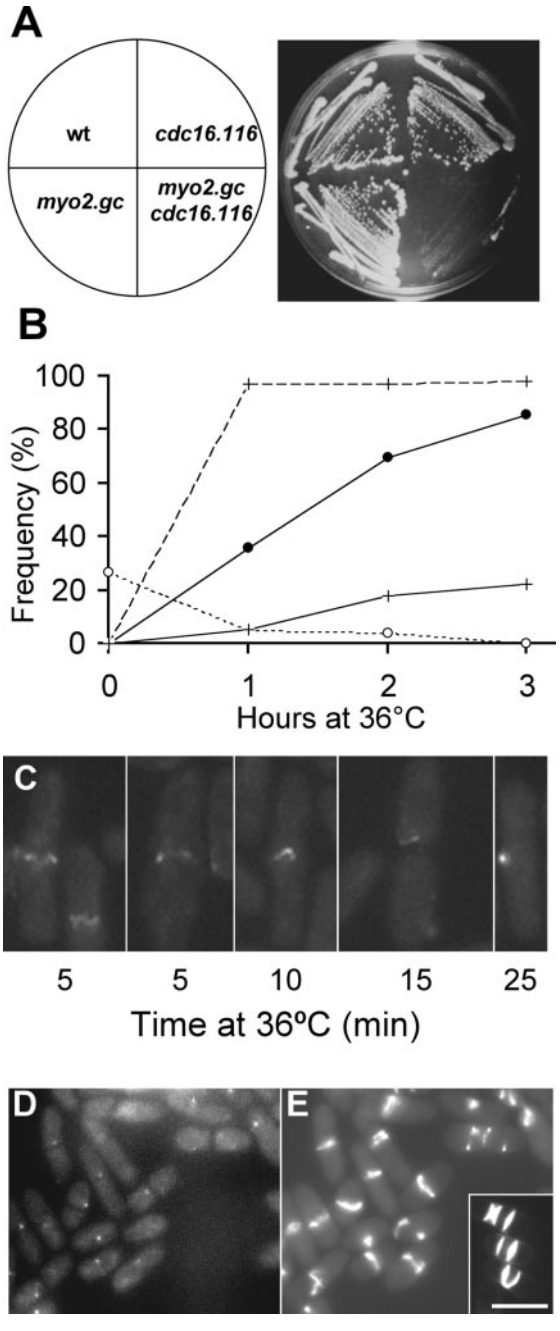


Figure 7. Cdc16 is required for the maintenance of the CAR. *myo2-gc cdc16-116* cells were unable to grow at 29°C, a temperature that permitted growth in strains bearing either allele individually (A). *myo2-gc cdc16-116* cells were grown to log phase at 25°C and then cultured at 36°C. Samples were taken at hourly time points and fixed to allow changes in the proportion of cells with Myo2-GFP rings (○, dotted line), contracted Myo2-GFP rings (●, solid line), septa (+ with dashed line), and aberrant septa (+ with solid line) to be examined (B). The proportion of cells containing Myo2-GFP rings declined over time. Rings assembled but subsequently collapsed (C) and the population of cells arrested with a single spot of Myo2-GFP fluorescence associated with septal material (D and E). *cdc16-116* cells in a *myo2*$^+$ background at the same 3-h time point are shown in the inset to E. The majority of cells had more than one morphologically normal septum. Bar, 10 μm.

1998) and the full story of the relationship between Myo2 and its presumptive kinase awaits further study.

CAR contraction in living fission yeast cells has been followed with the use of GFP-tagged Myo2 and Myp2 (Kitayama *et al.*, 1997; Bezanilla *et al.*, 2000; Mulvihill *et al.*, 2000) and by Balasubramanian *et al.* (1997) who made a GFP fusion protein with the Myo2 light chain Cdc4 (McCollum *et al.*, 1995, 1999). In none of these cases was the GFP reporter under the control of the endogenous promoter, moreover, cells retained the endogenous *myo2*$^+$ gene. We therefore created the strain *myo2-gc* in which the chromosomal copy of *myo2*$^+$ is fused to GFP and thus produces only the chimeric protein at endogenous levels. *myo2-gc* showed normal growth and cytokinesis in all growth conditions tested and mated normally. Hence, we were able to introduce the mutation into a variety of genetic backgrounds. *myo2-gc* was synthetically lethal with mutants in two of the SIN kinases, *cdc7-24* and *sid2-25* at the semipermissive temperature. We were unable to generate the double mutant *myo2-gc sid1-25* and we interpret this as indicating a very strong genetic interaction between these two alleles. That *myo2-gc*, in which GFP is fused to the C terminus of Myo2, has a cryptic cytokinesis phenotype is perhaps not surprising given the importance of this region of the tail for Myo2 localization (this report). Raising *myo2-gc cdc7-24* and *myo2-gc sid2-25* to the restrictive temperature had a striking effect on Myo2 organization; rings disappeared at 36°C as they did when GFP-Myo2 was expressed from a plasmid.

In theory, this simple yet novel assay of ring stability can be used to monitor, in living cells, the effect of any potential regulator of cytokinetic actomyosin ring formation for which a mutation is available. This is shown particularly clearly in the case of *cdc16-116* in which the SIN pathway is hyperactivated. Whereas *cdc16-116* in a *myo2*$^+$ background formed multiple septa as described originally by Minet *et al.* (1981), in *myo2-gc cdc16-116* cells mostly failed to form more than one septum and the morphology of the septa formed was markedly aberrant. Such cells were found to contain a single tight spot of GFP fluorescence. Closer analysis revealed that the CAR was unstable in this background and the spot of GFP-Myo2 was the final stage of CAR collapse. Taken together, our results suggest a model in which the assembly of the CAR and its subsequent contraction is under the control of the SIN pathway. We envisage that recruitment of Myo2 to the CAR is positively regulated by phosphorylation by both Cdc7 and Sid2, whereas CAR ring contraction is regulated by other SIN kinases and phosphatases. Based on their structural interdependence at the spindle poles, Guertin *et al.* (2000) envisage that the SIN proteins form a simple linear pathway with the order Cdc16/Byr4, Spg1, Cdc7, Sid1, and Sid2 that activates the cell wall β-glucan Cps1 (Balasubramanian *et al.*, 2000). We envisage the SIN proteins forming a complex in which the components interact in a variety of ways and which modifies a number of substrates, one of which is Myo2. The cytokinesis defect of *cdc7* mutants is likely due to the failure to phosphorylate S1518; this inhibits CAR formation and septation is consequently abolished. Myo2S^{1518}E forms rings n the absence of Cdc7 function but these are unable to contract and other modifications must take place to initiate this process. *cdc7* mutants form actin ring (Le Goff *et al.*, 1999), suggesting that

actin and Myo2 are brought to the CAR by different mechanisms.

The SIN pathway in fission yeast closely corresponds to the mitotic exit network in budding yeast (Hoyt, 2000). Lippincott *et al.* (2001) recently demonstrated a role for the TEM1 GTPase (the homolog of Spg1 in *S. pombe)* in CAR dynamics in budding yeast. The relationship between mitosis and cytokinesis is very different in the two yeasts. Nevertheless, a role for the SIN/mitotic exit network pathway in the control of CAR formation and function appears to be conserved. Our findings do not exclude the possibility that Myo2 is phosphorylated at other sites by protein kinases other than those in the septation pathway. In *Dictyostelium*, myosin II is phosphorylated by a myosin heavy chain specific protein kinase C (Matto-Yelin *et al.*, 1997) and the fission yeast protein kinase C homolog Pck2 localizes to the division site at cytokinesis (Sayers *et al.*, 2000). Myo2 is not regulated by phosphorylation of conserved sites in its essential light chain Cdc4 (McCollum *et al.*, 1999) and the regulatory light chain Rlc1 lacks the serine at amino acid position 19 that is associated with the regulation of Myo2 function in nonmuscle cells (Bresnick, 1999). As noted by others (Bresnick, 1999; Prokopenko *et al.*, 2000), it is likely that several signaling pathways converge on cytokinesis. Fission yeast are emerging as a powerful tool in which to dissect some of these processes.

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