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Evidence for F-actin-dependent and -independent mechanisms involved in assembly and stability of the medial actomyosin ring in fission yeast

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Cell division in a number of eukaryotes, including the fission yeast *Schizosaccharomyces pombe*, is achieved through a medially placed actomyosin-based contractile ring. Although several components of the actomyosin ring have been identified, the mechanisms regulating ring assembly are still not understood. Here, we show by biochemical and mutational studies that the *S.pombe* actomyosin ring component Cdc4p is a light chain associated with Myo2p, a myosin II heavy chain. Localization of Myo2p to the medial ring depended on Cdc4p function, whereas localization of Cdc4p at the division site was independent of Myo2p. Interestingly, the actin-binding and motor domains of Myo2p are not required for its accumulation at the division site although the motor activity of Myo2p is essential for assembly of a normal actomyosin ring. The initial assembly of Myo2p and Cdc4p at the division site requires a functional F-actin cytoskeleton. Once established, however, F-actin is not required for the maintenance of Cdc4p and Myo2p medial rings, suggesting that the attachment of Cdc4p and Myo2p to the division site involves proteins other than actin itself.

Keywords: Cdc4p/cytokinesis/Myo2p/myosin II/*S.pombe*

Introduction

Cytokinesis in a number of eukaryotic cells depends on an actomyosin-based contractile ring. It has been proposed that the constriction of the actomyosin ring provides the forces necessary for cell cleavage (Satterwhite and Pollard, 1992). In recent years, many protein components of the actomyosin ring have been identified from studies in a number of experimental systems (Satterwhite and Pollard, 1992; Gould and Simanis, 1997). However, many key aspects of the process are not fully understood, including the positioning of the cleavage plane, the reactions necessary for the assembly of the actomyosin ring and the coordination of the onset of cytokinesis with the completion of mitosis.

The fission yeast *Schizosaccharomyces pombe* is an attractive model organism to study cytokinesis. Cells of *S.pombe* divide using an F-actin ring that forms at mitosis and constricts during septum deposition (Marks and Hyams, 1985; Jochova *et al*., 1991). Recently, Myo2p/Rng5p (referred to as Myo2p) and Myp2p/Myo3p/Myo22p (hereafter referred to as Myp2p), two proteins related to myosin II heavy chains, have been identified as components of the contractile ring (therefore, we refer to the ring as an actomyosin ring) (Bezanilla *et al*., 1997; Kitayama *et al*., 1997; May *et al*., 1997, 1998; Motegi *et al*., 1997; Balasubramanian *et al*., 1998). Of these, Myo2p has been demonstrated to be essential for cytokinesis (Kitayama *et al*., 1997; May *et al*., 1997), while Myp2p is essential for cytokinesis only at lower temperatures and under stress conditions (Bezanilla *et al*., 1997; Motegi *et al*., 1997). Thus, as in other eukaryotes, the function of an actomyosin ring is essential for cytokinesis in *S.pombe*.

Genetic screens have identified mutations in at least nine genes that affect actomyosin ring positioning and assembly (Nurse *et al*., 1976; Chang *et al*., 1996; Sohrmann *et al*., 1996; Balasubramanian *et al*., 1998). *mid1/dmf1* is a gene that is essential for division site specification. Mid1p is localized in nuclei in interphase cells and in a medial ring in mitotic cells (Sohrmann *et al*., 1996), consistent with the proposal that the interphase nucleus positions the actomyosin ring in *S.pombe* (Chang *et al*., 1996). The genes *cdc3*, *cdc4*, *cdc8*, *cdc12* (Nurse *et al*., 1976), *rng2* (Chang *et al*., 1996; Balasubramanian *et al*., 1998), *rng3*, *rng4* and *rng5* (Balasubramanian *et al*., 1998) are essential for actomyosin ring assembly. The *cdc3* gene encodes the actin-binding protein profilin (Balasubramanian *et al*., 1994), and the *cdc12* gene encodes a formin-related protein (Chang *et al*., 1997). The *cdc4* gene encodes an EF-hand protein with properties of myosin light chain (McCollum *et al*., 1995), and the *cdc8* gene encodes the F-actin-binding protein tropomyosin (Balasubramanian *et al*., 1992). Recently, we have shown that the *rng2* gene encodes a protein related to mammalian IQGAP1 (Hart *et al*., 1996; Bashour *et al*., 1997), an actin- and calmodulin-binding protein, which is a potential effector of the RHO family of GTPases (Eng *et al*., 1998). The intracellular distribution patterns and the molecular identities of the gene products required for actomyosin ring assembly are consistent with the idea that these proteins interact to effect proper actomyosin ring formation. However, the steps and intermediates in the assembly of the actomyosin ring have remained elusive.

In this study, we have analysed the role of a type II myosin heavy chain Rng5p/Myo2p in actomyosin ring assembly in *S.pombe*. We show that Cdc4p, which we previously predicted to be a novel myosin light chain (McCollum *et al*., 1995), is indeed a light chain associated with Myo2p. Analysis of Myo2p and Cdc4p localization in cells treated with inhibitors of actin polymerization combined with structure–function studies of Myo2p have led us to identify certain F-actin-dependent and F-actin-independent steps in the assembly and maintenance of the medial actomyosin ring.

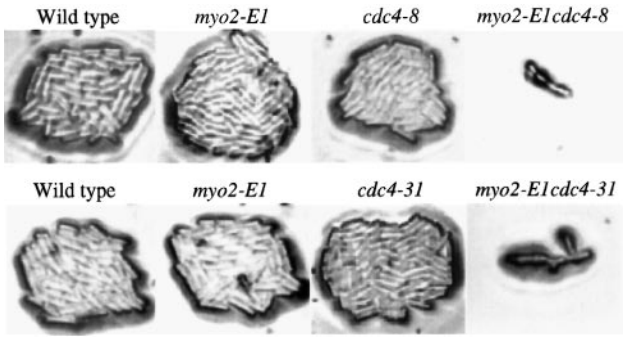

Fig. 1. Genetic interactions between *cdc4* and *myo2* mutants. Tetrads were dissected from crosses between *cdc4*-8 and *myo2*-E1, or *cdc4*-31 and *myo2*-E1, and incubated at 24°C.

Results

Genetic interactions between myo2 and cdc4

We have shown recently that the *S.pombe rng5* gene (Balasubramanian *et al*., 1998) is allelic with *myo2* (Kitayama *et al*., 1997; May *et al*., 1997), which encodes a myosin II heavy chain. Temperature-sensitive (ts) mutations in the *rng5* gene affect actomyosin ring assembly and impair cytokinesis (Balasubramanian *et al*., 1998). To identify genetic interactions involving *rng5/myo2*, we crossed the ts *myo2*-E1 mutant with other mutants defective in actomyosin ring assembly. We found that *myo2*-E1 showed a strong synthetic lethal interaction with the *cdc4*-8 and *cdc4*-31 mutations (Nurse *et al*., 1976). Under conditions in which *myo2*-E1, *cdc4*-8 and *cdc4*-31 single mutants were capable of colony formation, *myo2*-E1 *cdc4*-8 and *myo2*-E1 *cdc4*-31 double mutants arrested as elongated single cells incapable of cell division (Figure 1).

Cdc4p is a light chain associated with Myo2p

Previous work has shown that the *cdc4* gene encodes an EF-hand protein related to myosin light chains and is essential for cytokinesis in *S.pombe* (McCollum *et al*., 1995). To characterize further the suggested interaction between Myo2p and Cdc4p, antibodies specific to Myo2p (referred to as AbCD5) were raised. These antibodies recognized a single protein of M_r 190 kDa when lysates prepared from wild-type cells were immunoblotted (Figure 2A). The intensity of this signal and signals from presumed degradation products of Myo2p increased considerably when lysates prepared from cells overproducing Myo2p were immunoblotted (Figure 2A, lane OP2), confirming that the antibodies were specific to Myo2p. The failure of pre-immune serum to recognize *S.pombe* proteins upon immunoblotting (Figure 2A) in wild-type cells and in cells overproducing Myo2p further established that the 190 kDa protein detected by AbCD5 was indeed Myo2p. Finally, specificity of AbCD5 was demonstrated by the fact that the 190 kDa protein was not detected when proteins from germinating *myo2::ura4* spores were immunoblotted (data not shown).

Previous studies utilizing green fluorescent protein (GFP)-tagged Myo2p have shown that GFP–Myo2p was a component of the actomyosin ring in cells undergoing mitosis and cytokinesis, and was detected in a medial dot-like structure in some G_2 cells (Kitayama *et al*., 1997). Whereas pre-immune serum produced no specific staining, the use of AbCD5 in immunofluorescence experiments

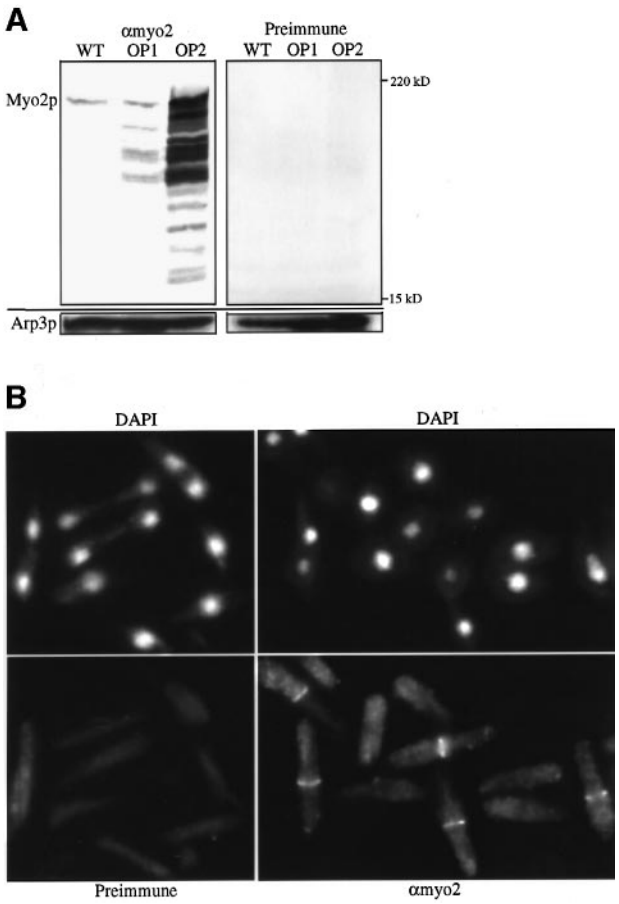

Fig. 2. Characterization of Myo2p antibodies by (**A**) immunoblot analysis and (**B**) immunofluorescence microscopy. For immunoblot analysis, denatured cell lysates were prepared from wild-type (WT) cells, and cells expressing Myo2p from a repressible promoter under conditions of promoter repression (OP1) and derepression (OP2). Lysates were separated by gel electrophoresis, immunoblotted and probed with antibodies against Myo2p (AbCD5; labelled as αmyo2) or pre-immune serum. Blots were reprobed with antibodies against Arp3p to ensure that approximately equal amounts of protein from the various strains had been loaded. For immunofluorescence microscopy, wild-type cells were fixed with methanol and stained with AbCD5 (αmyo2) or pre-immune serum, followed by Texas red-conjugated secondary antibodies, and mounted in DAPI mounting medium, to detect simultaneously the intracellular distribution of Myo2p and DNA.

showed localization of Myo2p in a medial ring in wild-type cells undergoing mitosis and cytokinesis (Figure 2B). However, a medial dot-like structure was not detected in interphase cells stained with AbCD5. GFP–Myo2p dots have been visualized only in cells expressing plasmid-borne $myo2^+$ fused to the gene encoding GFP (Kitayama *et al*., 1997; N.Naqvi and M.K.Balasubramanian, unpublished observations). It is possible that the increased amount of GFP–Myo2p permits its detection in the medial dot.

To address if Cdc4p was a light chain associated with Myo2p, non-denaturing lysates were prepared from wild-type cells and immunoprecipitated with antibodies specific to Myo2p or with antibodies specific to Cdc4p. Immune complexes subsequently were studied by immunoblot analysis using Myo2p or Cdc4p antibodies. Cdc4p antibodies precipitated a 190 kDa protein that was recognized by Myo2p antibodies upon immunoblotting (Figure 3A).

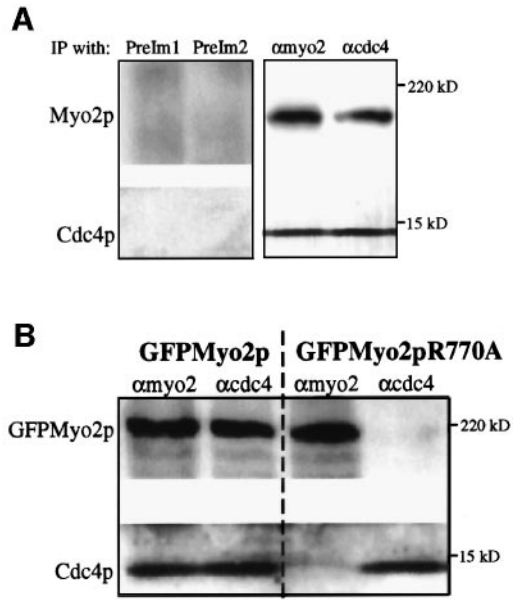

Fig. 3. Cdc4p is a light chain associated with Myo2p. (**A**) Myo2p and Cdc4p are present in a complex *in vivo*. Non-denaturing lysates prepared from wild-type cells were immunoprecipitated with Myo2p pre-immune serum (PreIm1), Cdc4p pre-immune serum (PreIm2), Myo2p antisera (αmyo2) and Cdc4p antisera (αcdc4). Immune complexes were resolved by electrophoresis and blotted to PVDF membranes. The portion of the blot above 46 kDa was probed with Myo2p antibodies and the portion below 46 kDa was probed with Cdc4p antisera. (**B**) Myo2p physical interaction with Cdc4p requires an intact IQ sequence motif. Non-denaturing lysates prepared from *myo2::ura4* mutants expressing GFP–Myo2p or GFP–Myo2R770A (IQ mutant) were immunoprecipitated with antibodies against Myo2p (αmyo2) and Cdc4p (αcdc4). Immune complexes were studied by immunoblotting with Myo2p and Cdc4p antibodies as described in (A).

Conversely, immune complexes generated with Myo2p antibodies were found to contain Cdc4p (Figure 3A). The co-immunoprecipitation studies established that Myo2p and Cdc4p are associated *in vivo*. However, it was unclear if Cdc4p was a light chain associated with Myo2p or if it was part of a multi-protein complex that contained Myo2p. Myosin heavy chains are known to bind their light chains through a sequence motif termed the IQ domain (Uyeda and Spudich, 1993; Zang *et al*., 1997). If Cdc4p were indeed a myosin light chain, mutation of the IQ domain(s) in Myo2p should render it incapable of binding Cdc4p. A canonical IQ sequence motif (first IQ motif; amino acids 765–775) and a less well conserved IQ sequence motif (second IQ sequence motif; amino acids 791–813) have been detected in the amino acid sequence of Myo2p (Kitayama *et al*., 1997; May *et al*., 1997). An essential arginine residue (Bezanilla *et al*., 1997; Kitayama *et al*., 1997) located within the first IQ domain of Myo2p (R770) was replaced with an alanine residue by site-directed mutagenesis to test if this IQ motif was important for binding to Cdc4p. The second IQ domain was not tested since it lacked this invariant arginine residue. Since a GFP–Myo2p fusion protein had been shown to be fully functional (Kitayama *et al*., 1997), we performed the mutagenesis study on the corresponding tagged *myo2* gene. This allowed us to assess if Cdc4p was a myosin light chain, as well as determine the intracellular distribution of the mutant, GFP–Myo2R770A. Interestingly, GFP–Myo2R770A was found to be capable of rescuing a *myo2::ura4* mutant, and the localization of Myo2R770A and Cdc4p was identical to that of the respective wild-type proteins (data not shown; see Discussion).

Myo2p or Cdc4p antibodies were used to immunoprecipitate the respective proteins from non-denaturing lysates prepared from the strains *myo2::ura4* pREP81GFP-Myo2R770A and *myo2::ura4* pREP81GFP-Myo2p (control). Again, immune complexes were studied by Western blot analysis with antibodies specific to Myo2p or Cdc4p. In these experiments, we found that GFP–Myo2p, like the untagged Myo2p, associated with Cdc4p (Figure 3B). However, GFP–Myo2R770A was found to be incapable of associating with Cdc4p (Figure 3B), although the mutant protein (GFP–Myo2R770A) was recognized and precipitated by AbCD5. Thus we conclude that Cdc4p is a light chain of Myo2p.

Cdc4p–Myo2p interactions and dependencies

In order to understand further the cellular role of the Myo2p–Cdc4p complex, we assessed the interactions between Myo2p and Cdc4p in two different assays. First, we asked if the interaction between Cdc4p and Myo2p was impaired in ts mutants in Myo2p or Cdc4p. To this end, *myo2*-E1, *cdc4*-8 and *cdc4*-31 mutants were grown at the permissive temperature to exponential phase and shifted to the restrictive temperature. Wild-type cells were also treated similarly to rule out effects of temperature shift on Myo2p–Cdc4p interactions. At 4 h after the temperature shift, cell lysates were prepared and immunoprecipitated with antibodies against Cdc4p. Immune complexes were immunoblotted and probed with antibodies against Myo2p or Cdc4p (Figure 4A). This analysis showed that the interaction between Cdc4p and Myo2p was not substantially altered in *myo2* and *cdc4* mutants.

Secondly, we addressed the dependencies between Cdc4p and Myo2p in achieving their localization to the division site. To resolve this issue, spores were prepared from diploid strains heterozygous for the *myo2*- or the *cdc4*-null alleles. Spore germination was carried out in medium lacking uracil such that only the spores bearing the *cdc4::ura4* or the *myo2::ura4* alleles would be capable of uracil metabolism and growth. Germinated spores were fixed and stained with antibodies against Cdc4p or Myo2p. Cdc4p was detected in improperly formed medial rings in cytokinesis-defective *myo2::ura4* spores, suggesting that the localization of Cdc4p to the division site did not require the function of Myo2p. However, Myo2p was not detected at the division site in germinating *cdc4::ura4* spores. Staining of the same cells with antibodies against tubulin revealed the presence of a mitotic spindle, thus confirming that the lack of Myo2p staining was not due to inefficient permeabilization of the germinated *cdc4::ura4* spores. Thus, we conclude that the assembly of Myo2p at the division site requires Cdc4p function, whereas assembly of Cdc4p at the division site does not require Myo2p function.

Myosin accumulation and assembly at the division site requires F-actin

Having established the dependencies of Cdc4p localization on Myo2p function and vice versa, we analysed the role of F-actin in localization of Myo2p and Cdc4p to the division site. The actomyosin ring is assembled in a

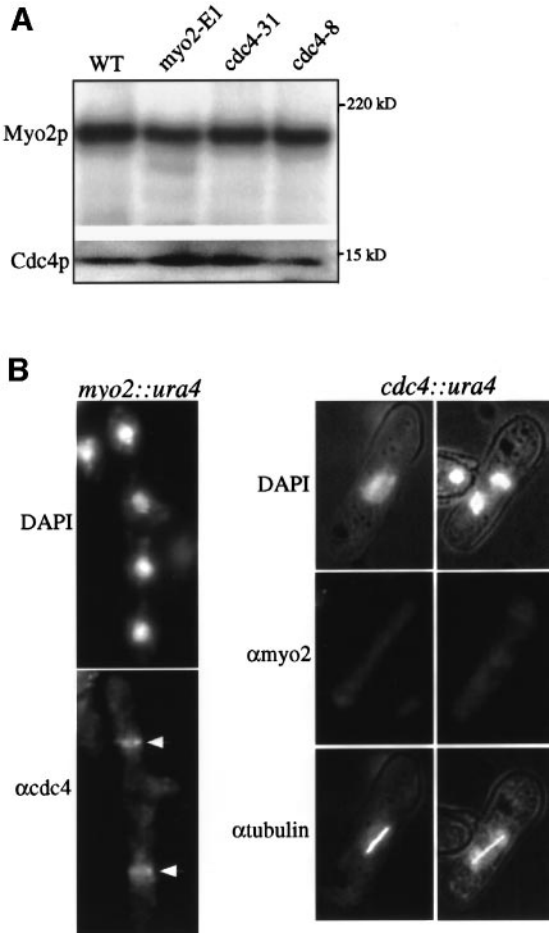

Fig. 4. Characterization of Myo2p–Cdc4p interactions. (**A**) Binding of Cdc4p to Myo2p is not disrupted in *cdc4* and *myo2* ts mutants. Non-denaturing lysates were prepared from wild-type cells (WT) and *cdc4*-8, *cdc4*-31, *myo2*-E1 mutants arrested at 36°C for 4 h, and immunoprecipitated with antibodies against Cdc4p. Immune complexes were analysed by immunoblotting with antibodies against Myo2p or Cdc4p as described in Figure 3A. (**B**) Cdc4p localization at the division site does not require Myo2p, whereas Myo2p assembly at the division site requires Cdc4p. Spores bearing null mutations in *myo2* (*myo2::ura4*) and *cdc4* (*cdc4::ura4*) were germinated in minimal medium lacking uracil, fixed and stained with antibodies against Myo2p (for the *cdc4::ura4* germination experiment) and Cdc4p (for the *myo2::ura4* germination experiment). Following antibody staining, germinated spores were mounted in DAPI to allow visualization of chromosomes. In addition, germinated *cdc4::ura4* spores were stained with antibodies against tubulin to ensure that these spores were permeable to antibodies.

spatially and temporally regulated manner in the medial region of the cell after entry into M phase. Therefore, we first addressed whether F-actin was required for the accumulation and assembly of Myo2p and Cdc4p at the division site. To this end, *cdc25*-22 cells (Russell and Nurse, 1986) were arrested at the G_2/M boundary by shifting to 36°C (restrictive temperature) for 3 h. Either latrunculin A (Lat-A), a drug that prevents actin polymerization (Ayscough *et al*., 1997), or dimethylsulfoxide (DMSO; solvent) were added to the arrested cultures which were incubated further at 36°C for 1 h and subsequently returned to 24°C (permissive temperature). At the time of return to 24°C, F-actin was not detected in the culture treated with Lat-A, but was detected at the growing ends of cells treated with DMSO (data not shown). At 90 min

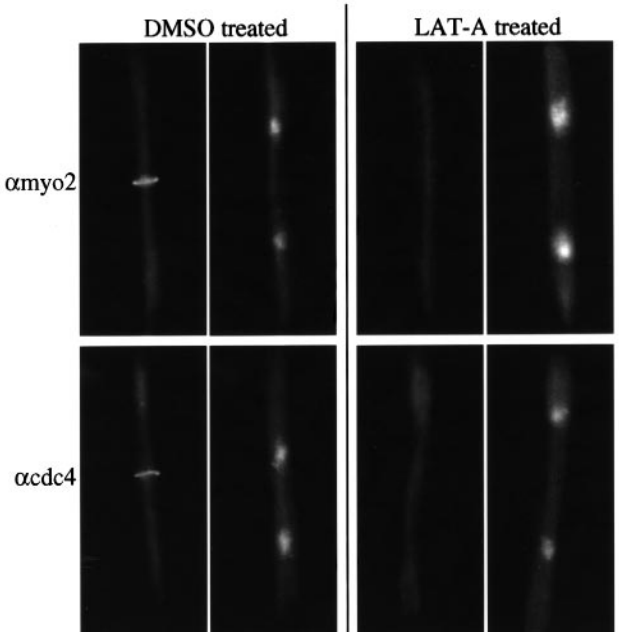

Fig. 5. F-actin is required for accumulation of Myo2p and Cdc4p at the division site. *cdc25*-22 cells were arrested at the restrictive temperature (36°C) for 3 h. The culture was split two ways and Lat-A was added to one and DMSO (control) was added to the other. Cells were incubated further at (36°C) for 1 h and then returned to the permissive temperature (24°C). When the majority of cells were judged to be in anaphase, cells were fixed with methanol and stained with antibodies against Myo2p (αmyo2) or Cdc4p (αcdc4), and with DAPI to visualize the nuclei.

after return to 24°C, the majority of cells treated with DMSO had entered mitosis and were binucleate. In these binucleate cells, Cdc4p and Myo2p were detected in medial rings (Figure 5). Cells treated with Lat-A entered mitosis, albeit more slowly than control cells, but Cdc4p and Myo2p were not detected in the medial region of cells (Figure 5). Immunoblotting experiments (data not shown) revealed that Myo2p and Cdc4p were stable in cells treated with Lat-A, and that the absence of F-actin did not cause degradation of Myo2p and Cdc4p. These studies established that the accumulation and assembly of the Myo2p–Cdc4p complex at the division site during mitosis requires F-actin.

Maintenance of the myosin ring after its assembly does not require F-actin

Next, we asked whether F-actin is required for integrity of the ring structure(s) containing Myo2p and Cdc4p. *Schizosaccharomyces pombe* cells expressing GFP–Cdc4p and GFP–Myo2p were treated for 30 min with Lat-A or with DMSO as a control. F-actin was only detected in cells treated with DMSO, and Myo2p and Cdc4p, as expected, were detected in rings in all cells passing through mitosis and cytokinesis. Interestingly, Myo2p and Cdc4p rings were visualized in all mitotic cells treated with Lat-A (data not shown). These experiments suggested that Myo2p and Cdc4p were anchored to the division site independently of F-actin.

To establish clearly whether F-actin was required for maintenance of Myo2p rings in a synchronous population of cells, we arrested the *nda3*-KM311 mutant at its restrictive temperature. Under these conditions, *nda3*-

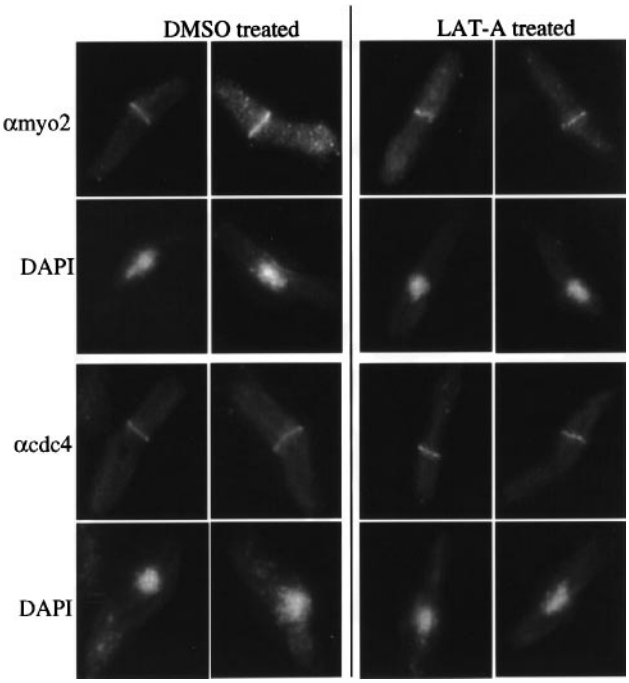

Fig. 6. F-actin is not required for integrity of Myo2p and Cdc4p rings. An exponential culture of *nda3*-KM311 was shifted to the restrictive temperature of 20°C for 10 h and then incubated further for 30 min in the presence of DMSO or Lat-A. Cold-arrested cells were fixed with methanol and stained with antibodies against Myo2p (αmyo2) or Cdc4p (αcdc4). After antibody stainings, cells were resuspended in DAPI to allow visualization of DNA.

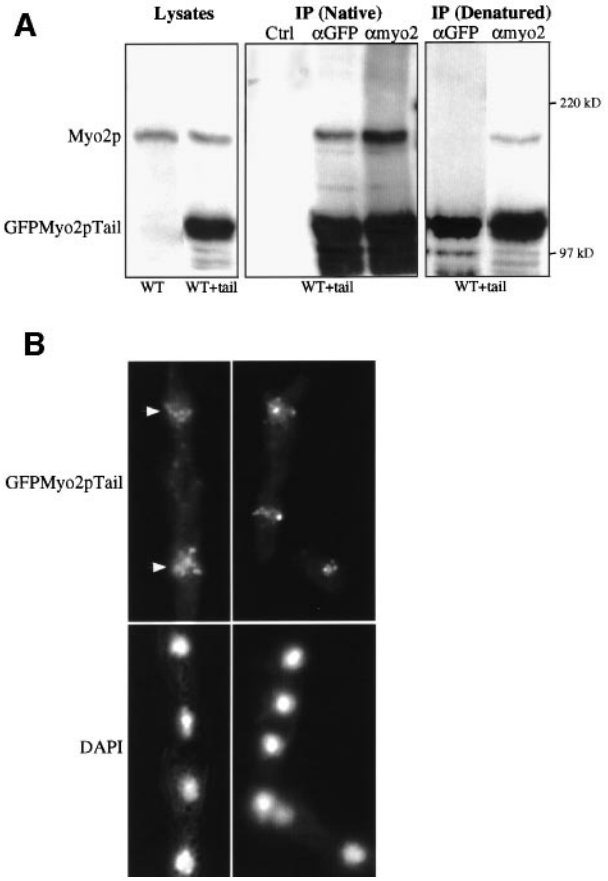

Fig. 7. (**A**) Intermolecular interactions between Myo2p tails. Cell lysates (native or denatured) were prepared from wild-type cells expressing GFP–Myo2tail (WT+tail) and immunoprecipitated with antibodies against GFP (αGFP) or with AbCD5 (αmyo2). Immune complexes were resolved by electrophoresis, blotted and probed with antibodies against Myo2p. In addition, cell lysates were immunoblotted to ascertain the position of Myo2p (190 kDa) and GFP–Myo2tail (110 kDa). Negative control (ctrl) refers to the immunoprecipitation performed with non-specific rabbit immunoglobulins. (**B**) Myo2p tail is sufficient for localization to the division site. Germinating *myo2::ura4* spores expressing GFP–Myo2tail were fixed and stained with DAPI. Arrowheads indicate the GFP–Myo2tail aggregates as spots or patches at the division sites.

KM311 mutant cells block in mitosis, without a mitotic spindle, but with a normally assembled actomyosin ring (Hiraoka *et al*., 1984; Chang *et al*., 1996). Arrested *nda3*-KM311 cells were treated for 30 min with Lat-A or with DMSO as a control, fixed and stained with antibodies against Cdc4p or Myo2p. Disappearance of F-actin upon Lat-A treatment was confirmed by staining with rhodamine-conjugated phalloidin. Myo2p and Cdc4p were detected in rings in cells treated with DMSO, as well as those treated with Lat-A, suggesting that the stability of Myo2p and Cdc4p rings did not depend on the presence of F-actin (Figure 6). Similar results were also obtained when the cold-arrested *nda3*-KM311 mutant was treated with Lat-A for longer periods. Thus, we conclude that the stability of the Myo2p and Cdc4p rings does not require F-actin function.

Myo2p tail interacts with intact Myo2p

Previous studies have demonstrated the importance of the tail region of the *Dictyostelium* myosin II in its assembly into filaments (Moores and Spudich, 1998). Given that the tail of Myo2p contains proline residues (Kitayama *et al*., 1997), which are known to break α-helices, we wished to know if *S.pombe* Myo2p formed filaments. We have been unable to obtain the tail region of Myo2p in a soluble form to assess its ability to form heat-stable coiled-coil structures in the presence of high salt, as demonstrated for other coiled-coil proteins such as Cdc8p (Balasubramanian *et al*., 1992). As an alternative, therefore, we addressed the interaction between Myo2p tails by co-immunoprecipitation experiments. To this end, a DNA molecule which encoded the tail of Myo2p fused to GFP (referred as GFP–Myo2tail) was constructed and introduced into a wild-type strain. GFP–Myo2tail lacks amino acids 1–778, which includes the actin-binding domain, the ATP-binding domain and the first IQ domain. Lysates were prepared from cells expressing GFP–Myo2tail and immunoblotted. A 190 and a 110 kDa protein was detected in the strain carrying the plasmid. The 110 kDa, but not the 190 kDa, protein was absent when lysates from wild-type cells lacking GFP–Myo2tail were immunoblotted, confirming that the 110 kDa protein is GFP–Myo2tail (Figure 7A). Immunoprecipitation experiments were carried out under native and denaturing conditions using antibodies against Myo2p or against GFP. As shown in Figure 7A, immune complexes prepared under native conditions with either GFP antibodies or Myo2p antibodies contained both Myo2p and GFP–Myo2tail. Under denaturing conditions, immune complexes prepared with Myo2p antibodies contained Myo2p and GFP–Myo2tail. However, immune complexes prepared with GFP antibodies under denaturing conditions

only contained GFP–Myo2tail and did not contain Myo2p. Although these experiments do not provide evidence for filament formation, they establish the intermolecular interaction between Myo2p mediated by its tail sequences, perhaps allowing assembly of Myo2p tails into filaments.

To determine the localization of GFP–Myo2tail, a plasmid encoding this mutant Myo2p version was introduced into a strain with the relevant genotype *myo2::ura4/myo2*$^{+}$. The diploid transformant was sporulated and haploid spores were selected on medium lacking uracil (to select for the myosin-null mutation) and leucine (to select for the plasmid expressing GFP–Myo2tail). Spores expressing GFP–Myo2tail were defective in cytokinesis, although DNA replication and mitosis were not affected. Thus, these germinating spores accumulated multiple nuclei before they lysed. Interestingly, GFP–Myo2tail was detected at the division site as spots and patches (Figure 7B). This experiment suggested that the actin-binding domain, the ATPase domain and the first IQ motif of Myo2p are not important for accumulation of Myo2p at the division site.

Myo2p ATP-binding site mutants do not assemble a normal actomyosin ring

Previous studies have shown that cells lacking Myo2p accumulate improperly organized F-actin rings and improper septa (Kitayama *et al*., 1997; May *et al*., 1997; Balasubramanian *et al*., 1998). This led to a possibility that Myo2p was required as an actin-binding protein essential for actomyosin ring assembly. Alternatively, a normal actomyosin ring might be assembled and aligned by the forces produced by interactions between F-actin and myosin II (Satterwhite and Pollard, 1992). To distinguish between these possibilities, the core (amino acids 173–177, sequence GAGKT) of the motor domain in Myo2p, responsible for ATP binding, was replaced with alanines (amino acids 173–177, sequence AAAAA). Again, the mutagenesis was performed on the gene encoding a GFP–Myo2p fusion protein. A plasmid expressing the ATPase domain mutant Myo2p defective in binding ATP (referred to as GFP–Myo2ΔATP) was introduced into a diploid strain of the genotype *myo2::ura4/myo2*$^{+}$. This diploid transformant was sporulated and haploid spores were selected on medium lacking uracil and leucine. As expected, spores expressing GFP–Myo2ΔATP failed to proliferate, establishing that ATP binding was essential for *S.pombe* Myo2p function. To characterize further the terminal phenotype, spores expressing GFP–Myo2ΔATP were fixed and stained to detect F-actin. Spores expressing GFP–Myo2ΔATP were capable of germination, cell elongation, DNA replication and mitosis, but were impaired for cytokinesis and accumulated multiple nuclei, before they lysed (Figure 8). In germinating spores undergoing first and second mitosis, GFP–Myo2ΔATP was detected at the division site during mitosis as improperly organized cable-like structures, or in dot-like structures (Figure 8). Similarly, F-actin localization (Figure 8) was aberrant and unusual, and normal looking actomyosin rings were not observed in these germinating spores undergoing mitosis. These experiments suggested that the assembly of a functional actomyosin ring depends on the motor activity of Myo2p.

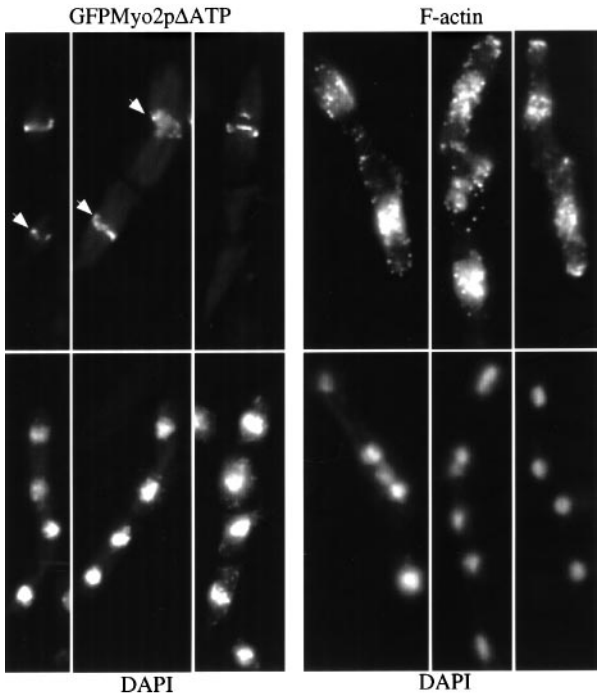

Fig. 8. Defective actomyosin ring assembly in a Myo2p mutant altered in its ATP-binding site. Germinating *myo2::ura4* spores expressing GFP–Myo2ΔATP were fixed with formaldehyde and stained with antibodies against GFP or a monoclonal antibody against actin. After antibody staining, cells were resuspended in DAPI to allow visualization of DNA. The improper rings formed by GFP–Myo2ΔATP are highlighted with arrowheads.

Discussion

Myo2p and Cdc4p constitute a myosin heavy chain–light chain pair

Our previous study had identified the product of the *S.pombe cdc4* gene as an EF-hand protein, which is a component of the actomyosin ring and is essential for cytokinesis (McCollum *et al*., 1995). Furthermore, it was demonstrated that Cdc4p binds a 190 kDa protein with ATP-binding activity (McCollum *et al*., 1995). These observations led to the suggestion that Cdc4p is a myosin light chain. However, the molecular identity of the putative myosin heavy chain was unknown. In this study, we have demonstrated that Cdc4p binds Myo2p, a 190 kDa protein containing an ATPase domain with similarity to myosin II heavy chains. Myosins are known to bind their light chains through the IQ domains (Uyeda and Spudich, 1993; Zang *et al*., 1997). The physical interactions between Myo2p and Cdc4p and dependence of this interaction on the IQ domain suggest that Cdc4p is indeed a light chain associated with Myo2p.

A well conserved IQ domain (first IQ domain) and a less well conserved IQ domain (second IQ domain) have been detected in the primary sequence of Myo2p (May *et al*., 1997). The first IQ domain typically binds the myosin essential light chain, whereas the second IQ domain binds the myosin regulatory light chain (May *et al*., 1997; Zang *et al*., 1997). Since interaction between Cdc4p and Myo2p is abolished by a point mutation in the first IQ domain, it is likely that Cdc4p functions in a manner similar to myosin essential light chains. Presently, it is unclear if some other light chain binds Myo2p at the second IQ site.

Cells expressing a Myo2p IQ domain mutant, which does not show a detectable interaction with Cdc4p, are viable. One possibility is that the Cdc4p–Myo2R770A interaction is weak enough to be detected by immunoprecipitation, but that this residual binding (if any) is sufficient to allow function of the Cdc4p–Myo2R770A complex *in vivo*. However, it is also possible that Myo2p normally functions together with its light chain (i.e. Cdc4p), but that the mutation in the IQ domain relieves the dependency of Myo2p function of its interaction with Cdc4p. A similar dependency relationship between heavy and light chains has been demonstrated in previous studies of type I and type II myosins (Uyeda and Spudich, 1993; Zang *et al*., 1997; Geli *et al*., 1998).

The F-actin ring assembly defect of *cdc4*-null mutants (McCollum *et al*., 1995) is stronger than that of *myo2*-null mutants (Kitayama *et al*., 1997). Thus, it is possible that Cdc4p interacts with more than one protein involved in actomyosin ring assembly and/or stability. Consistent with this, we have shown that Cdc4p is localized to the improperly organized F-actin rings in *myo2*-null mutants (Figure 4) and in the normal actomyosin rings in *myo2::ura4* mutants rescued by GFP–Myo2R770A, which is defective in interaction with Cdc4p (data not shown). A likely candidate for another interacting protein is Myp2p. However, given that a *myo2*Δ*myp2*Δ mutant makes F-actin rings, although improperly (Motegi *et al*., 1997), and that *cdc4*-null mutants do not assemble any F-actin rings (McCollum *et al*., 1995), it is unlikely that Myp2p represents the only other target of Cdc4p. The budding yeast Mlc1p, a protein related to Cdc4p, recently has been identified as a light chain associated with the type V myosin, Myo2p (Stevens and Davis, 1998). Further studies of Cdc4p should identify whether Cdc4p also interacts with unconventional myosins.

A pathway for actomyosin ring assembly involving F-actin and Myo2p

The experiments presented herein have identified distinct steps that might occur in the assembly of the actomyosin ring. The actomyosin ring is formed in a spatially and temporally controlled manner in the medial region of the cell after entry into the M phase (Marks and Hyams, 1985; Snell and Nurse, 1994). Block and release experiments performed on *cdc25*-22 mutants in the presence or absence of an inhibitor of actin polymerization suggest that F-actin is required for the accumulation and assembly of Myo2p and Cdc4p at the division site. F-actin is detected in *S.pombe* in three major structures; patches at regions of cell wall/septum deposition, medial rings in cells undergoing mitosis and cytokinesis, and in cables that run the long axis of the cells (Marks and Hyams, 1985; Balasubramanian *et al*., 1996). We and others (Arai *et al*., 1998; M.K.Balasubramanian, unpublished results) have found recently that the *S.pombe* tropomyosin, Cdc8p, is a component of these cables in addition to being part of the medial actomyosin ring and some patch-like structures (Balasubramanian *et al*., 1992; Arai *et al*., 1998). Furthermore, in mitotic cells, F-actin cables appear to be linked to the medial actomyosin ring (Arai *et al*., 1998). Thus, it is likely that Myo2p and Cdc4p are transported along these cables to the division site. Since mutant Myo2p expressing only its tail can still accumulate in the medial region of the cell, it is possible that the tail of Myo2p binds molecules involved in the F-actin-based accumulation of Myo2p at the division site. Isolation and characterization of mutants which only affect F-actin cables, but not patches, will be useful in assessing if transport along F-actin cables is important for accumulation of ring components at the division site.

F-actin-dependent accumulation and assembly of Myo2p and Cdc4p at the division site in *S.pombe* is different from the F-actin-independent assembly of the Myo1p (a type II myosin heavy chain) ring in *Saccharomyces cerevisiae* (Bi *et al*., 1998). Interestingly, the assembly of Myo1p and the IQGAP-related protein Iqg1p (Epp and Chant, 1997; Lippincott and Li, 1998; Osman and Cerrione, 1998) at the cytokinetic ring in *S.cerevisiae* is dependent on septins (Bi *et al*., 1998; Lippincott and Li, 1998). Previously, we have shown that assembly of the *S.pombe* IQGAP-related protein, Rng2p, at the cytokinetic ring depends on F-actin (Eng *et al*., 1998). Thus, it appears that F-actin is important for both assembly and constriction of the actomyosin ring in *S.pombe*, whereas F-actin is required only for the constriction of the cytokinetic ring in *S.cerevisiae*.

Localization of Myo2p to the division site is abolished in *cdc4::ura4* mutants, whereas Cdc4p is detected in improperly organized medial rings in *myo2::ura4* mutants. These irregular Cdc4p rings were also observed in *myo2::ura4* mutants expressing GFP–Myo2tail or GFP–Myo2ΔATP (data not shown). Presently, it is unclear whether the failure to detect Myo2p in *cdc4::ura4* mutants is due to a direct consequence of the lack of Cdc4p, or an indirect effect of the inability of *cdc4::ura4* cells to make a medial ring containing F-actin. Surprisingly, however, mutant Myo2p lacking its head and the IQ domain still assembles at the division site. Perhaps molecules that anchor Myo2p aggregates and stabilize them are dependent on Cdc4p for their own localization.

Our structure–function analyses of Myo2p have provided further insights into the mechanism of actomyosin ring assembly. Myo2p expressing only its tail accumulates in the medial region of the cell, suggesting that the F-actin-binding domain, the ATPase domain and the first IQ motif are not important for its localization to the division site. We interpret results obtained from spore germination experiments with caution, since some maternal wild-type Myo2p might be present in *myo2::ura4* spores obtained from the heterozygous strain *myo2*$^{+}$/*myo2::ura4*. However, given that the tail of *Dictyostelium* myosin II can accumulate at the division cortex in the form of aggregates (Zang and Spudich, 1998), and that *S.pombe* Myo2p lacking its tail does not localize to the division site (N.Naqvi and M.K.Balasubramanian, unpublished observations), it is likely that the tails of type II myosins are important for their localization to the site of cell cleavage. Normal actomyosin rings were not assembled in *myo2::ura4* mutants expressing a Myo2p mutant defective in its ATP-binding site. In these germinating spores, both F-actin and Myo2p were detected at the region between dividing nuclei in the form of cables and patches, which had apparently failed to assemble into a continuous ring. Thus, the role of Myo2p as a motor might be important in organizing the actomyosin ring.

Forces generated by actomyosin interaction might align F-actin cables and Myo2p aggregates into a functional ring.

Following assembly of normal actomyosin rings, F-actin is not required for integrity of the pre-existing Myo2p/Cdc4p ring(s). Based on the disappearance of F-actin structures after brief treatment with Lat-A, it has been proposed that actin undergoes rapid cycles of polymerization and depolymerization in yeast cells (Ayscough *et al*., 1997). Thus, the stability of the actomyosin ring might depend on the association of proteins other than actin itself with the plasma membrane. Perhaps Myo2p and/or Cdc4p fulfil such a role.

Further work will be required to clarify some issues relating to the assembly and function of the actomyosin ring. (i) Given that Myo2p/Cdc4p rings are retained in cells depleted of F-actin, is there an interaction between Myo2p/Cdc4p and the plasma membrane and if so what is its molecular nature? (ii) Following ring assembly, does Myo2p play a role in constriction of a properly organized actomyosin ring, as has been proposed from studies in higher eukaryotes (Satterwhite and Pollard, 1992)? Although our studies provide evidence for intermolecular interaction between Myo2p tails, it will be important to determine by biochemical methods and electron microscopy if *S.pombe* Myo2p forms bipolar thick filaments. (iii) Given that Cdc4p is detected in improperly organized medial rings in cells lacking Myo2p, what are the other targets of Cdc4p and what role(s) do these targets play in cytokinesis?

Materials and methods

Yeast strains, media and genetic methods

The genotypes of the *S.pombe* strains used in this study are: *cdc25*-22 *ura4*-D18 h^+ (MBY166), *leu1*-32 *ura4*-D18 (MBY192), *cdc4*-8 *ade6*-210 *ura4*-D18 h^+ (MBY24), *cdc4*-31 *ade6*-210 *leu1*-32 h^+ (MBY25), *myo2*-E1 *ade6*-216 *ura4*-D18 *leu1*-32 h^- (MBY151), *nda3*-KM311 *ura4*-D18 h^- (MBY389), *myo2::ura4* pREP81::GFP–Myo2, *myo2::ura4* pREP81::GFP–Myo2R770A, *myo2::ura4* pREP81::GFP–Myo2tail and *myo2::ura4* pREP81::GFP–Myo2ΔATP. Media and genetic methods were as described (Moreno *et al*., 1991). Yeast transformation was carried out by electroporation (Prentice, 1992). Lat-A (Molecular Probes) was used at 100 μM concentration. Thiamine was used at a final concentration of 5 μM to repress transcription from the *nmt1* promoter (Maundrell, 1989). Genetic crosses were performed at 28°C on YPD agar plates. After 2 days, tetrads were dissected using a Singer MSM micromanipulator. Terminal phenotypes of mutants were examined following growth on YE agar medium at the appropriate temperature(s).

Recombinant DNA techniques

All DNA manipulations and bacterial transformations were performed according to standard techniques (Sambrook *et al*., 1989). For expression studies of the complete *myo2* open reading frame (ORF), an *Nde*I site was introduced at the initiator methionine using PCR amplification with the primers MOH52 (5′-TAT GAC AGA AGT AAT ATC TAA TAA AAT AAC TGC AAA AGA TGG TGC AA-3′) and MOH 53 (5′-CTA GTT GCA CCA TCT TTT GCA GTT ATT TTA TTA GAT ATT ACT TCT GTC A-3′). The complete *myo2* coding region was then cloned into the pREP1 vector (Maundrell, 1993) at the *Nde*I–*Sma*I site using a tripartite ligation involving a 0.27 kb *Nde*I–*Pst*I fragment and a 4.7 kb *Pst*I–*Sma*I fragment to give plasmid pCDL205. Plasmid pCDL207 and pCDL252 were constructed in the same way but in pREP42::GFP and pREP81::GFP backgrounds, respectively. For mutating the IQ domain in *myo2*, the invariant arginine residue in this motif at position 770 was replaced with an alanine residue by site-directed mutagenesis of pCDL252 using the U.S.E™ kit (Pharmacia) with primer 5′-PCA-AACAAGGATCGCTGGCTTTCTTCAAAGAAA-3′ to generate the GFP–Myo2R770A. Primer 5′-PGGTGAGTCCGCGGCAGCTGCAGC-AGAGAATACC-3′ was utilized similarly to make GFP–Myo2ΔATP by replacing amino acids 173–177 (GAGKT) with AAAAA. The region encoding amino acids 1–778 was deleted from $myo2^+$ and the remainder of the ORF expressed as a GFP fusion to generate the GFP–Myo2tail (this represents the tail region of Myo2p fused to the GFP moiety).

Antibodies and immunoprecipitations

A 1.1 kb *Hin*dIII fragment encoding the C-terminal 350 codons of $myo2^+$ was expressed as a fusion protein with GST (Smith and Johnson, 1988) in *Escherichia coli*. Purified GST–Myo2p was used as an antigen to raise antibodies against Myo2p. Immunoblot analysis of total cell lysates prepared under denaturing conditions from *S.pombe* cells demonstrated that the antisera thus generated recognized a specific polypeptide with an apparent mol. wt of 190 kDa. SDS–PAGE (6–18%) analysis for protein separation was performed as described (Laemmli *et al*., 1970). Detections for the Western analyses were done by the chemiluminescent method using secondary antibodies conjugated to peroxidase. Primary antisera (αMyo2p or αCdc4p) was used at a 1:400 dilution. Wash buffer was Tris-buffered saline containing 0.05% Tween-20. Total cell extracts (non-denaturing) from *S.pombe* strains were made in the NP-40 buffer, and the relevant conditions and methodology used for immunoprecipitation experiments have been described previously (McCollum *et al*., 1995). Lysates prepared under denaturing conditions were according to Moreno *et al*. (1991) and diluted 1:15 with NP-40 buffer when such lysates were used in immunoprecipitation experiments.

Fluorescence microscopy

F-actin staining was performed as described (Balasubramanian *et al*., 1997) using cells fixed for 1 min with formaldehyde. Immunostainings with Myo2p and Cdc4p antibodies were carried out on methanol-fixed cells as described (Balasubramanian *et al*., 1997). Texas red- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Molecular Probes) were used for detection of bound antibodies. Cells were viewed using a Leica DMLB microscope and images captured using an Optronics DEI-750T cooled CCD camera. Images were acquired using the Leica Qwin software and processed using the Adobe Photoshop program.

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