Molecular mechanism of myosin-II assembly at the division site in

*Schizosaccharomyces pombe*

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SUMMARY

*Schizosaccharomyces pombe* cells divide by virtue of the F-actin-based contractile ring (F-actin ring). Two myosin-II heavy chains, Myo2 and Myp2/Myo3, have been localized to the F-actin ring. Here, we investigated the mechanism of myosin-II assembly at the division site in *S. pombe* cells.

First, we showed that Cdc4, an EF-hand protein, appears to be a common myosin light chain associated with both Myo2 and Myo3. Loss of function of both Myo2 and Myo3 caused a defect in F-actin assembly at the division site, like the phenotype of *cdc4* null cells. It is suggested that Myo2, Myo3 and Cdc4 function in a cooperative manner in the formation of the F-actin ring during mitosis.

Next, we investigated the dynamics of myosin-II during mitosis in *S. pombe* cells. In early mitosis when accumulation of F-actin cables in the medial region was not yet observed, Myo2 was detected primarily as dots widely located in the medial cortex. Myo2 fibers also became visible following the appearance of the dots. The Myo2 dots and fibers then fused with each other to form a medial cortical network. Some Myo2 dots appeared to be localized with F-actin cables which are also accumulated in the medial region. Finally these structures were packed into a thin contractile ring. In mutant cells that cannot form the F-actin ring such as *cdc3(ts), cdc8(ts)* and *cdc12(ts)*, Myo2 was able to accumulate as dots in the medial cortex, whereas no accumulation of Myo2 dots was detected in *cdc4(ts)* cells. Moreover, disruption of F-actin in the cell by applying latrunculin-A did not affect the accumulation of Myo2 dots, suggesting that F-actin is not required for their accumulation.

A truncated Myo2 which lacks putative Cdc4-binding sites (Myo2dIQs) was able to rescue *myo2* null cells, *myo3* null cells, *cdc4(ts)* mutant cells and *cdc4* null cells. The Myo2dIQs could assemble into a normal-shaped ring in these cells. Therefore, its assembly at the division site does not require the function of either Cdc4 or Myo3.

Key words: *Schizosaccharomyces pombe*, Myosin-II, Actin, Contractile ring, Cytokinesis

INTRODUCTION

Cytokinesis in many eukaryotic cells takes place through contraction of the contractile ring which is mainly composed of F-actin (reviewed by Schroeder, 1975; Mabuchi, 1986). Myosin-II localizes in the cleavage furrow (Fujiwara and Pollard, 1976) and is believed to be a molecular motor that generates a force for contraction of the contractile ring through interaction with F-actin (Mabuchi and Okuno, 1977; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). Localization of myosin-II changes dynamically during mitosis: it is diffusely distributed in the prometaphase cytoplasm. During metaphase to anaphase, it is recruited to the medial cortex which then constricts to form a cleavage furrow (DeBiasio et al., 1996; Neujahr et al., 1997).

To understand the molecular process of myosin-II assembly in the cleavage furrow, molecular genetical approaches have been taken using *Dictostelium discoideum* amoeba. Studies with mutant myosin-IIs have indicated that the formation of bipolar thick filaments is crucial for proper localization of myosin-II in the cleavage furrow: the mutant myosin-IIs that cannot form thick filaments in vitro fail to assemble in the cleavage furrow (Egelhoff et al., 1993; Sabry et al., 1997). On the other hand, both the phosphorylation of regulatory light chain (RLC) at the myosin light chain kinase (MLCK) site and the motor activity of myosin-II are not essential for the assembly of myosin-II in the cleavage furrow (Ostrow et al., 1994; Uyeda and Spudich, 1993; Y umura and Uyeda, 1997a,b; Zang et al., 1997). Recently, Zang and Spudich (1998) have shown that the tail region of myosin-II without both motor domain and actin-binding site is able to move toward the cleavage furrow. In contrast, the phosphorylation state of RLC dramatically changes during mitosis in vertebrate cells (Satterwhite et al., 1992; Yamakita et al., 1994; Matsumura et al., 1998). RLC phosphorylation at the MLCK site seems to be concentrated in the middle region of the cell during cytokinesis (Matsumura et al., 1998; Murata-Hori et al., 1998). These studies suggest that RLC phosphorylation may be important
for localization of myosin-II in the cleavage furrow in vertebrate cells. However, it is not yet clear whether this is the case or not. There could be a common mechanism other than phosphorylation for myosin-II to assemble in the cleavage furrow.

The fission yeast Schizosaccharomyces pombe is an excellent organism to study cytokinesis at a molecular level. Like most animal cells, the actomyosin-based contractile ring (F-actin ring) is formed in the medial cortex of the mitotic cell (Marks and Hyams, 1985; Kanbe et al., 1989; Kitayama et al., 1997), although invagination of the primary septum follows the contraction of the F-actin ring in S. pombe (reviewed by Gould and Simanis, 1997). Two myosin-II heavy chains (myosin-II HCs), Myo2 and Myo3/Myo3, have been identified as components of the F-actin ring (Kitayama et al., 1997; May et al., 1997; Bezanilla et al., 1997; Motegi et al., 1997; Balasubramanian et al., 1998). Myo2 is essential for cell growth, while Myo3 is conditionally essential. We found that Myo2 and Myo3 function in a cooperative manner in the formation of the F-actin ring (Motegi et al., 1997). Genetic screenings have identified genes and their products in the formation of the F-actin ring (Motegi et al., 1997; May et al., 1997; Bezanilla et al., 1997; Motegi et al., 1997). Genetic screenings have identified genes and their products that are necessary for the assembly of the F-actin ring in S. pombe (reviewed by Gould and Simanis, 1997). Two genes, plo1+, which encodes a homologue of polo kinase and cdc15+, which encodes a PSTPIP family protein, seem to act at the initiation of F-actin ring formation, since overexpression of either of these genes leads to F-actin ring formation even in interphase cells (Ohkura et al., 1995; Fankhauser et al., 1995). The cdc3+, cdc4+, cdc8+, cdc12+ and rng2+ genes are essential for assembly of the F-actin ring (Nurse et al., 1976; Chang et al., 1996; Eng et al., 1998). All of these gene products are localized to the F-actin ring. cdc3+ and cdc8+ mutant cells display delocalized F-actin structures in all steps of the cell cycle. The cdc3+ gene encodes profilin, which is thought to induce polymerization of F-actin (Balasubramanian et al., 1993). The cdc8+ gene encodes tropomyosin (Balasubramanian et al., 1992) which localizes to both F-actin cables and the F-actin ring and seems to be required for stabilization of these structures (Arai et al., 1998). cdc12+, rng2+ and cdc4+ mutant cells showed abnormal F-actin accumulation only during mitosis, indicating that these genes may play specific roles in F-actin ring assembly. The cdc12+ gene encodes a diaphanous family protein (Chang et al., 1997) whose function is required for cytokinesis in other organisms (Castrillon and Wasserman, 1994; Watanabe et al., 1997; Swan et al., 1998). The rng2+ gene encodes an IQGAP family protein (Eng et al., 1998), which has an essential role in cytokinesis in S. cerevisiae and D. discoideum (reviewed by Machesky, 1998). The cdc4+ gene encodes an EF-hand protein similar to myosin light chain (McCollum et al., 1995). Actually, Cdc4 has recently been shown to associate with Myo2 (Naqvi et al., 1999). Therefore, factors which may function in the assembly of F-actin as the F-actin ring are being classified. In contrast, very little is known about the assembly of myosin-II at the division site.

In the present study, we investigated the mechanism of myosin-II assembly at the division site using S. pombe cells. First, we analyzed the function of the two myosin-II heavy chains in the formation of the F-actin ring. Next, we investigated how myosin-II assemblies to form the ring structure during mitosis. Moreover, the role of F-actin and Cdc4 in the localization of myosin-II during mitosis was investigated.

**MATERIALS AND METHODS**

**Strains and genetic techniques**

The genotypes of the S. pombe strains used in this study are h- (Y1), h- ade6-M216 leu1-32 ura4-D18 myo3::ura4+ (FM301), h- ade6-M216 leu1-32 ura4-D18 rng5/myo2-E1 (myo2-E1), h- ade6-M216 leu1-32 ura4-D18 rng5/myo2-E1 (myo2-E1), h- cdc25-22 leu1-32 (cdc25-22), h- ade6-M210 cdc3-124 (cdc3), h- leu1-32 ura4-D18 cdc4-8 (cdc4), ade6-M210 leu1-32 ura4-D18 cdc8-110 (cdc8), h- ura4-D18 cdc12-112 (cdc12), h- leu1-32 nda3-KM111 (nda3-KM111), h+/h0 ade6-M210/ad6-M216 leu1/leu1 ura4-D18/ura4-D18 myo2::ura4+myo2+ (XF572), and h- ade6-M216 leu1 ura4-D18 myo2::ura4+ (FM201). The media used were those described previously (Moreno et al., 1991). Standard procedures for S. pombe genetics were carried out according to the method of Alfa et al. (1993) and Moreno et al. (1991). Latrunculin-A (Lat-A) (Wako Pure Chem., Osaka, Japan) was used at 10 μM. Thiamine was used at 5 μM to repress the expression from the nmt1 promoter (Maundrell, 1989, 1993).

**DNA manipulation**

Standard methods of DNA manipulations were carried out according to Sambrook et al. (1989). To express green fluorescent protein (GFP)-fusion genes in the cell, pGFPV1 was used, which carries an enhanced version of GFP in the prEP81 that carries the thiamine-repressible nmt1 promoter (Maundrell, 1989, 1993). To express the entire myo3+ gene from the nmt1 promoter, nucleotides spanning from +3 to +3 of the gene (TTAATG) were replaced by the Ndel target sequence (CATATG) according to a standard protocol for site directed mutagenesis. The complete myo3+ coding region downstream of this Ndel site was cloned into pGFPV1. For deleting the IQ domains in myo2+ gene, two rounds of PCR were performed. Nucleotides spanning from 2268 to 2273 (ACTTATTA) and from 2454 to 2459 (AGTACT) were replaced by the Ndel target sequence, and two amplified PCR fragments were ligated at this Ndel site. The myo2+ sequence between the EcoRV and SacI sites was replaced by the mutated PCR fragment. This mutant myo2+ gene (myo2dIQs) was cloned into pGFPV1.

**Antibodies and immunoprecipitation**

A 1.6 kb EcoRI fragment encoding the tail region of Myo2 was expressed as a fusion protein with glutathione S-transferase (GST) in Escherichia coli. Purified GST-Myo2 was used as an antigen to raise antibodies against Myo2. It was emulsified with Freund’s complete adjuvant (Difco, Detroit, MI, USA) and injected subcutaneously into a male rabbit. Evidencing injections were carried out with the antigen emulsified with Freund’s incomplete adjuvant. After obtaining antisera, antibodies were purified through an affinity column in which the antigen was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden).

For immunoblot analysis, total cell extracts of S. pombe cells were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Proteins were first reacted with anti-Myo2 antibodies and then detected using secondary antibodies coupled to peroxidase.

Immunoprecipitation experiments were carried out according to the method of Arai et al. (1998). Affinity-purified antibodies against GFP (a-GFP mAb; Molecular Probes, Inc., Eugene, OR, USA), Cdc4 (a-Cdc4 pAb; a gift from Dr. Mohan K. Balasubramanian, National University of Singapore) and Myo2 (a-Myo2 pAb), respectively, were conjugated with Protein A-Sepharose beads (Sigma Chem. Co., St...
Louis, MO, USA). *S. pombe* cells suspended in F1 buffer (1 mM MgCl₂, 10 mM MOPS, pH 7.5, 1 mM dithiothreitol) containing 0.6 M KCl, 10 mM ATP, 0.1% Nonidet P-40 and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 20 μM benzamidine-HCl, 2 μg/ml o-phenanthroline and 5 μg/ml each of aprotinin, leupeptin, pepstatin A and aprotinin) were disrupted with glass beads. KCl and ATP were included to prevent possible aggregation and attachment to F-actin, respectively, of Myo2. The homogenates were centrifuged at 10,000 g for 20 minutes, and the supernatant was applied to the antibody-conjugated beads, and swirled at 4°C overnight. After washing the beads, proteins bound to the beads were eluted using 0.1 M glycine-HCl (pH 2.5), and the eluate was immediately neutralized. The eluted proteins were subjected to SDS-PAGE and immunoblotting.

### Fractionation of cell extracts

Cells were broken in F1 buffer containing 50 mM KCl and protease inhibitors, and the homogenates were spun at 1,000 g for 5 minutes at 4°C to remove unbroken cells. The supernatant was then spun at 100,000 g for 1 hour at 4°C. As described in Results, Myo2 was concentrated in the pellet fraction. In order to investigate solubility of Myo2, the pellet was resuspended in F1 buffer containing 5 mM ATP, 1% Triton X-100 or 0.5 M NaCl, and spun at 100,000 g for 1 hour at 4°C to obtain supernatant and pellet, which were then subjected to SDS-PAGE.

### Two-hybrid techniques

Two-hybrid assays were performed as described previously (Gyuris et al., 1993). Nucleotides 1998 to 2994 of the *cdc4* gene encoding IQ domains (Myo2IQ), nucleotides 2116 to 2924 of the *myo3* gene encoding IQ domains (Myo3IQ), and the full-length *cdc4* gene were amplified by PCR. Appropriate restriction sites were introduced with primers. Sequences to be tested were fused with the lex DNA-binding domain (DAD) in plasmid pBTM116 or with the activation domain (AD) in plasmid pGAD424. L40 strain containing lexAop-lacZ reporter gene was cotransformed with plasmid expressing lex DAD-fusion protein and with plasmid expressing AD-fusion protein. Interactions between these fusion proteins were monitored by β-galactosidase activities. A picture was taken at 8 hours after plating on an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing plate at 30°C.

### Fluorescence microscopy

Immunofluorescence staining of the *S. pombe* cells was carried out according to the method of Arai et al. (1998). Affinity-purified antibodies against Myo2, Cdc4 and GFP (Molecular Probes, Inc.) were used as primary antibodies. Secondary antibodies used were rhodamine- or bodipy (4, 4-difluoro-4-bora-3a, 4a diaza-s-Indacene)-conjugated goat IgG against rabbit IgG (Molecular Probes, Inc.). The specimens were finally stained with bodipy-phallacidin or rhodamine-phalloidin (Molecular Probes, Inc.), and 4',6-diamido-2-phenylindole (DAPI).

For time-lapse microscopic studies of living cells expressing GFP-Myo2, including observations by deconvolution microscopy, 1 μl of concentrated cell suspension were placed on a coverslip. Observations were performed at 20-22°C.

Fluorescent images were recorded using two approaches. Conventional two-dimensional images were recorded under a Zeiss Axioskop fluorescence microscope using a Plan Apochromat ×63 lens (Carl Zeiss, Inc., Oberkochen, Jena, Germany). Photographs were taken on Kodak T-Max ASA 400 films. Three-dimensional images were obtained using a Delta Vision system (Applied Precision, Issaquah, WA, USA) attached to an Olympus IX-70-SIF microscope equipped with a UplanApo ×100 Oil lens (Olympus, Tokyo, Japan). Images were captured with a cooled CCD camera (Photometrics, Munchen, Germany). Thirty 0.2 μm optical sections from top to bottom of a single *S. pombe* cell were recorded, and out-of-focus light was removed by iterative deconvolution with a Silicon Graphics (Mountain View, CA) IRIX work station. The ‘max intensity’ mode was used for calculation of fluorescence intensities according to the manufacturer’s instructions. Three-dimensional images were constructed from the deconvoluted two-dimensional images.

### RESULTS

#### Myosin-II HCs are required for the formation of the F-actin ring

To investigate the role of the two myosin-II HCs in the formation of the F-actin ring we analyzed a phenotype of *myo2 myo3* double mutant cells. Since *myo2* is an essential gene, but *myo3* is not, we created *myo2-E1 myo3 null (myo3Δ) cells* by disrupting *myo3* gene in *myo2-E1* temperature-sensitive mutant cells (Balasubramanian et al., 1998). The *myo2-E1 myo3Δ* cells showed a temperature-sensitive phenotype that was more severe than that of the single mutant cells (Fig. 1A). Both the *myo2-E1* cells and the *myo3A* cells could form colonies at temperatures between 25°C and 34°C. On the other hand, the *myo2-E1 myo3Δ* cells could not form colonies at 34°C, although they could form small colonies at temperatures between 25°C and 30°C. The *myo2-E1* cells showed a normal shape and normal distribution of F-actin throughout the cell cycle at 25°C (Fig. 1Ba,b), although they showed aberrant accumulation of F-actin between segregating nuclei during mitosis at 37°C (Fig. 1Bc,d). On the other hand, the *myo2-E1 myo3Δ* cells were unusually elongated, swollen and branched, and contained multinuclei, even at the permissive temperature (Fig. 1Be). F-actin patches were dispersed throughout the cell, and abnormal aggregates of F-actin cables were seen between the segregating nuclei (Fig. 1BF). At 37°C, F-actin patches were reduced and dispersed, and no F-actin accumulation was observed between segregating nuclei (Fig. 1Bg-h). Moreover, Cdc4 staining was not detected in these cells at 37°C (data not shown). These results suggest that the two myosin-II HCs, Myo2 and Myo3, are necessary for the assembly of the F-actin ring during mitosis.

#### Localization of Myo2 and Myo3 in mitotic cells

Both the two myosin-II HCs are components of the F-actin ring in *S. pombe*. However, the function of Myo2 might be different from that of Myo3, since *myo2* is an essential gene, but *myo3* is not. To verify the difference between the function of Myo2 and that of Myo3, we carried out an elaborate analysis on the localization of Myo2 and Myo3 during the cell cycle, particularly during mitosis, in a single *S. pombe* cell. To observe the localization of Myo2, we raised polyclonal antibodies against Myo2 (a-Myo2 pAb). Immunoblotting showed that the antibodies recognized a single protein of 190 kDa in an extract from wild-type cells (Fig. 2A). The intensity of this signal increased in an extract from cells overexpressing Myo2 (Fig. 2A). Proteins of lower molecular mass which were also recognized by a-Myo2 pAb in this extract may be degradation products of overexpressed-Myo2, since amounts of these polypeptides relative to the 190 kDa protein varied when the expression level of Myo2 was varied (data not shown). Pre-immune serum did not recognize any proteins in each extract. Thus, the 190 kDa protein recognized by the antibodies was considered to be Myo2. The staining with a-
Myo2 pAb also showed a medial ring in the mitotic wild-type cell, while the pre-immune serum showed no specific staining (data not shown). This result is consistent with previous studies (Kitayama et al., 1997; Naqvi et al., 1999). However, a spot-like structure of Myo2 which has been detected in cells expressing GFP-Myo2 (Kitayama et al., 1997) was not recognized in wild-type cells stained with a-Myo2 pAb (data not shown).

To observe the localization of Myo3, we created myo3Δ cells carrying plasmid pGFP81myo3. Immunoblotting showed that antibodies against GFP recognized a single protein of about 240 kDa in an extract from these cells, while they did not recognize any protein in an extract from the myo3Δ cells carrying pREP81myo3 (Fig. 2B). Thus, the 240 kDa protein was considered to be GFP-tagged Myo3. Expression of GFP-Myo3 rescued a temperature-sensitive defect of the myo3Δ cells (data not shown), indicating that the fusion protein is functional in the cell. Fluorescence of GFP was seen as spots during interphase or as a medial ring during mitosis in the cells under conditions inducing transcription of GFP-Myo3 (Fig. 2Cc), but no fluorescence was observed in the cells under conditions suppressing the expression (data not shown). Thus, the GFP fluorescence we detected in the cells is due to the functional GFP-Myo3 fusion protein.

To investigate the localization of both Myo2 and Myo3 in the same specimen, the myo3Δ cells carrying pGFP81myo3 were stained with a-Myo2 pAb. The staining pattern of Myo2 seemed to be the same as the fluorescence pattern of GFP-Myo3 in mitotic cells (Fig. 2Ca-c). During early mitosis, Myo2 and GFP-Myo3 appeared at the division site almost at the same time (Fig. 2C, arrows). In 100% (135/135) of the early mitotic cells displaying a GFP-Myo3 ring at the division site, Myo2 colocalized to the ring. In 90% (111/123) of the early mitotic cells showing a Myo2 ring at the division site, GFP-Myo3 colocalized to the ring. About 10% (12/123) of the cells that showed the Myo2 ring did not show the GFP-Myo3 ring. This could be due to photo-bleaching of the GFP-Myo3, since the fluorescent signal of GFP-Myo3 was weaker than that of rhodamine-stained Myo2. Myo2 was sometimes localized to a GFP-Myo3 spot in interphase or mitotic cells (Fig. 2Cb,c). Therefore, the mode of assembly property of Myo3 in the cell may be similar to that of Myo2 during mitosis.

Cdc4 is a common MLC of both myosin-II HCs

To test for a physical interaction between Myo3 and Cdc4, we first conducted immunoprecipitation experiments. An extract enriched with GFP-Myo3 was used for immunoprecipitation with a-GFP mAb or with a-Cdc4 pAb. The immune complex precipitated with a-GFP mAb were found to contain both GFP-Myo3 and Cdc4 (Fig. 3A). Conversely, GFP-Myo3 was recovered in the Cdc4 immunoprecipitates (Fig. 3A). As a negative control, a similar experiment was performed using normal IgG for immunoprecipitation; neither of the proteins was detected in the immunoprecipitates (Fig. 3A). As a positive control, we used an extract enriched for GFP-Myo2, because it has been reported that Myo2 associates with Cdc4 (Naqvi et al., 1999). The interaction between GFP-Myo2 and Cdc4 was also confirmed under the same experimental conditions (Fig. 3A). As a negative control, a similar experiment was performed using normal IgG for immunoprecipitation; neither of the proteins was detected in the immunoprecipitates (Fig. 3A). 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Myosin-II localization in *S. pombe*

IQ-domains of Myo3 were sufficient for Cdc4-binding, as well as those of Myo2.

**Dynamics of Myo2 in mitotic cells**

We investigated the dynamics of Myo2 assembly during mitosis, because the function of Myo2 might be more important for cytokinesis than that of Myo3.

First, we investigated the distribution of Myo2 in wild-type cells during mitosis by immunofluorescence microscopy using a-Myo2 pAb. To visualize the distribution of Myo2 in detail, we reconstructed three-dimensional images from serial optical sections. The cell cycle stage of early mitotic cells was judged from the organization of F-actin cables (Arai et al., 1998; R. Arai and I. Mabuchi, unpublished). During interphase, F-actin patches were located at the ends of the cell and F-actin cables ran along the long axis of the cell (Fig. 4Aa). Myo2 was detected as sparse dots localized throughout the cell (Fig. 4Ab), especially in the cell cortex as seen in a cross-sectional view of the cell (Fig. 4Ad). During early mitosis when fine F-actin cables were randomly distributed throughout the cell (Fig. 4Ba), Myo2 was detected primarily as dots distributing in a relatively wide medial region (Fig. 4Bb). These dots were about 0.1-0.2 \( \mu m \) in diameter. All these dots were found to be located in the cortex in a cross-sectional view of the cell (Fig. 4Cb). The Myo2 dots did not seem to localize to the aster-like structure of the F-actin cables. Then, the network of F-actin cables was further developed at the wide medial region of the cell (Fig. 4Da). The fluorescence of Myo2...
dots near the division plane became larger and stronger. Some Myo2 dots possessed tail-like fibrous projections (Fig. 4Db, arrow). The dots and the projections tended to align along the F-actin cables (Fig. 4Dc, arrowheads). The continuous F-actin ring was established in the next stage in the division plane (Fig. 4Ea, arrows). Major Myo2 dots were aligned on the F-actin ring (Fig. 4Ec).

Next, the dynamics of Myo2 during mitosis and cytokinesis were investigated by observation of living cells expressing GFP-Myo2. Synchronously dividing cdc25-22 myo2Δ cells expressing GFP-Myo2 that had been released from arrest (Russell and Nurse, 1986) were used. Twenty four out of the 25 cells we observed formed a GFP-Myo2 ring during mitosis and accomplished cytokinesis under the experimental conditions, indicating that the time-lapse observations did not affect the normal division of the cell. GFP-Myo2 was detected as a few large spots in the cells arrested in G2/M phase, as described previously (Kitayama et al., 1997), which were not detected by immunofluorescence microscopy as described above. These spots positioned at the medial region of the cell at a very early stage of mitosis (Fig. 5, arrows). In a few minutes, small GFP-Myo2 dots, distinct from the large spots, appeared and increased in number and GFP-Myo2 fibers also become visible in the medial cortex. The dots seemed to be connected to the fibers, and these structures formed a network that surrounds the cell at the medial region (Fig. 5, arrowheads). The network, which was initially 3 to 4 μm in width, was then packed into a sharp ring within several minutes. The tightly-packed GFP-Myo2 ring then contracted to a point. The speed of the contraction was 0.7 to 0.8 μm/minute. We also observed similar dynamics for GFP-Myo2 in myo2Δ cells expressing GFP-Myo2: 12 out of the 12 cells we observed showed assembly of GFP-Myo2 similar to that observed in the synchronized cdc25-22 myo2Δ cells expressing GFP-Myo2 (data not shown).

Roles of contractile ring components and the mitotic spindle in the localization of Myo2
The localization of Myo2 in mutant cells defective in the formation of the F-actin ring was investigated. Immunoblotting studies showed that expression levels of Myo2 were normal in cdc3Δ, cdc4Δ, cdc8Δ, cdc12Δ, myo2-E1 and nda3-KM311 cells (data not shown). In mitotic cdc4Δ cells showing accumulation of disorganized F-actin cables at the middle region of the cell (McCollum et al., 1995; Chang et al., 1996), no significant Myo2 staining was seen (Fig. 6A). cdc3Δ or cdc8Δ cells showed delocalized F-actin patches during all steps of the cell cycle as described previously (Balasubramanian et al., 1992,
Myosin-II localization in S. pombe

1994; Chang et al., 1996; Arai et al., 1998). Neither F-actin cable nor F-actin ring was detected in these mutant cells. cdc12 ts cells showed normal F-actin distribution during interphase, but delocalized F-actin patches during mitosis as described by Chang et al. (1996, 1997). In 75% of mitotic cdc3 ts cells, 81% of mitotic cdc8 ts cells and 71% of mitotic cdc12 ts cells, the Myo2 dots appeared in the wide medial region of the cells (Fig. 6B,C,D). After 4.5 hours from the temperature-shift, these mutant cells contained 3 to 4 nuclei (51% of the cdc3 ts cells, 47% of the cdc8 ts cells and 49% of the cdc12 ts cells). These cells also showed the cortical Myo2 dots between the newly segregating nuclei (data not shown). Thus, it is suggested that F-actin cables and proper localization of F-actin patches were not required for the appearance of Myo2 dots at the medial cortex of the cell.

Next, we investigated a role of the mitotic spindle in the localization of Myo2 by examining a β-tubulin mutant nda3-KM311 cells (Hiraoka et al., 1984). 87% of nda3-KM311 cells arrested at the restrictive temperature showed a wide ring of F-actin, and tightly-packed Myo2 ring at the middle of the cells (Fig. 6E). Thus, it appears that the mitotic spindle is not required for the formation of the Myo2 ring.

We also stained cdc4 ts, cdc3 ts, cdc8 ts, cdc12 ts, and nda3-KM311 cells with antibodies against Cdc4. The Cdc4 localization was quite similar to that of Myo2 (data not shown).

**Fig. 6.** (A-E) Behavior of Myo2 in cell division mutants. cdc3 ts, cdc4 ts, cdc8 ts, and cdc12 ts cells were grown at a permissive temperature, and then shifted to the restrictive temperature for 4 hours. nda3-KM311 cells were grown at a permissive temperature, and then shifted to the restrictive temperature for 8 hours. The cells were simultaneously stained with DAPI (blue), bodipy-phallacidin (green) and antibodies against Myo2 (red). (a) F-actin stainings. (b) Myo2 stainings. (c) Merged images of DNA, F-actin and Myo2. (d) Localization of GFP-Myo2 in myo2Δ cdc3 ts, myo2Δ cdc8 ts, myo2Δ cdc12 ts, or myo2Δ nda3-KM311 cells (green). Pictures presented are 3-D reconstituted images. Bar, 3.3 μm.
To exclude the possibility that these staining patterns might be artificial due to the immunofluorescence technique used, we expressed GFP-Myo2 in myo2Δ cdc3ts, myo2Δ cdc8ts, myo2Δ cdc12ts, or myo2Δ nda3-KM311 cells. The fluorescence patterns of GFP-Myo2 in these double mutant cells were similar to those observed using the immunofluorescence technique (Fig. 6Bd,Cd,Dd,Ed).

myo2-E1 cells show accumulation of disorganized F-actin cables at the medial cortex during mitosis (Balasubramanian et al., 1998). We found that the dots of mutant Myo2 were localized to these F-actin cables in these cells (data not shown).

**F-actin is not required for accumulation of the Myo2 dots, but is required for formation of the Myo2 ring**

The above experiments indicated that proper F-actin organization and F-actin cables are not required for the accumulation of Myo2 dots at the medial cortex. It has been shown that a GFP-tagged tail domain of Myo2 that lacks actin-binding site is accumulated as spots at the division site, and maintenance of the Myo2 ring does not require F-actin after its formation (Naqvi et al., 1999). Thus, we expected that the accumulation of the Myo2 dots may occur independently of F-actin. We verified this hypothesis by observing cdc25-22 cells in which F-actin had been disrupted during G2/M arrest by using Lat-A (Ayscough et al., 1997). The cdc25-22 cells in 10 μM Lat-A could not grow, but the cells treated with 0.1% DMSO, solvent used for Lat-A, grew well (data not shown). The cells treated with 10 μM Lat-A for 5 minutes showed no F-actin staining, but DMSO-treated cells displayed a normal F-actin distribution (data not shown). The arrested cdc25-22 cells were incubated with 10 μM Lat-A for 10 minutes at 37°C, and then shifted to 25°C. At 1 hour after the release from the G2/M arrest, 87% of the cells treated with DMSO showed both the F-actin ring and the Myo2 ring (Fig. 7Aa-c). On the other hand, at 1.5 hours after the release, 74% of the cells treated with Lat-A displayed accumulation of Myo2 dots at the medial cortex despite no staining of F-actin being observed (Fig. 7Ad-e). To further confirm these results, we used cdc25-22 myo2Δ cells expressing GFP-Myo2 in the same experiment. Though 85% of the DMSO-treated cells showed both the F-actin and the GFP-Myo2 ring (Fig. 7Bg), 82% of the Lat-A treated cells displayed the GFP-Myo2 dots in the medial cortex (Fig. 7Bh). Even at 2 hours after the release, the Lat-A treated cells could not show either fibrous or ring distribution of GFP-Myo2 (data not shown). Therefore, it is suggested that F-actin is not required for the accumulation of Myo2 dots in the medial cortex.

**Solubility of Myo2 changes during the cell cycle**

We investigated localization of Myo2 by fractionation of synchronous cdc25-22 cells released from the G2/M boundary. Immediately after entry into mitosis, Myo2 was found both in a soluble fraction (S) and in a pellet fraction (P) (Fig. 8B). At 1 to 1.5 hours after the release when the cells formed the F-actin ring, Myo2 was found only in P (Fig. 8B). At 2 hours after the release when the septum was formed, Myo2 was again found both in S and in P (Fig. 8B). In an extract of arrested nda3-KM311 cells that showed a wide F-actin ring (Hiraoka et al., 1984; Chang et al., 1996), Myo2 was found primarily in P (data not shown). Thus, it is suggested that aggregation of Myo2 or association of Myo2 with some proteins or organelles fractionated in P might have occurred during the formation of the F-actin ring. Because actin was fractionated exclusively in P (Fig. 8B), we examined whether Myo2 might form a rigor-
like association with F-actin. A part of Myo2 was solubilized from the P obtained from mitotic cells by the addition of 5 mM Mg^{2+}-ATP (Fig. 8C). In either the extract of the cells before the formation of the F-actin ring or that of cells possessing the F-actin ring, about half of the Myo2 was extracted from the P fraction (Fig. 8C).

**Truncated Myo2 lacking its IQ domains is able to assemble at the division site in myo2Δ cells, cdc4Δ cells and cdc4ΔΔ cells**

Although Cdc4 is required for the accumulation of Myo2 at the division site as described above, it is not clear how Cdc4 contributes to the proper localization of Myo2. To investigate a role of Cdc4 in the regulation of Myo2 localization, we first analyzed a function of mutant Myo2 that lacks putative Cdc4-binding domains. A heterozygous diploid was transformed with pGFP81myo2dIQs, which expresses a GFP-Myo2 lacking both of the two IQ-domains. The transformed diploid was sporulated and subjected to random spore analysis. The myo2Δ spores were able to form colonies under conditions that induce expression of GFP-Myo2dIQs. Immunoblotting showed that these cells did not express endogenous Myo2 (Fig. 9A). Thus, Myo2 lacking the IQ domains rescued the lethality of the myo2Δ cells. Immunoprecipitation studies showed that an immune complex with a-Myo2 pAb from an extract of these cells did not contain Cdc4 (Fig. 9A), indicating that the deletion of IQ-domains of Myo2 caused a loss of binding of Cdc4. A majority of myo2Δ cells expressing GFP-Myo2dIQs (62%) showed a normal morphology, and GFP-Myo2dIQs assembled into a medial ring in these cells (Fig. 9Cc, arrows). However, the growth rate of these cells was slower than that of myo2Δ cells expressing GFP-Myo2 (Fig. 9B), and the rest of the cells (38%) showed more or less elongated morphology and contained three to four nuclei, indicating a defect in cytokinesis. Therefore, although GFP-Myo2dIQs can assemble into a ring, the ring sometimes may not be able to contract effectively. In some cells which completed septation, aberrant cables of GFP-Myo2dIQs were seen at the septation site (Fig. 9Cc, arrowhead). To test a possibility that GFP-Myo2dIQs may be transported to the division site by direct or indirect association with Myo3, we created myo2Δ myo3Δ cells expressing GFP-Myo2dIQs. GFP-Myo2dIQs also assembled into a medial ring in these cells (data not shown), indicating that Myo3 is not involved in the assembly of GFP-Myo2dIQs into the ring.

We next analyzed an effect of GFP-Myo2dIQs expression under the loss of function of Cdc4. cdc4Δ myo2Δ cells expressing GFP-Myo2 grew well at 25°C, but could not grow at 37°C (Fig. 9Dc). cdc4Δ myo2Δ cells expressing GFP-Myo2dIQs could grow at 25 to 37°C (Fig. 9Db). Thus, GFP-Myo2dIQ was capable of suppressing the temperature-sensitive phenotype of the cdc4Δ cells. Moreover, cdc4Δ myo2Δ cells expressing GFP-Myo2dIQs were viable, although they showed swollen and elongated morphology and contained multinuclei (65%), indicating a defect in cytokinesis (Fig. 9E). However, both F-actin and GFP-Myo2dIQs assembled into a ring at the division site (Fig. 9Eb,c). Thus, the mutant Myo2 lacking putative Cdc4-binding sites is able to assemble as a medial ring under the loss of Cdc4 function, although it may have some problem in rendering full contractility to the contractile ring.

**DISCUSSION**

**Myosin-IIs composed of Myo2, Myo3 and Cdc4 are essential for the formation of contractile ring**

Two myosin-II HCs, Myo2 and Myp2/Myo3, have been identified in *S. pombe* (Balasubramanian et al., 1998; Bezanilla et al., 1997; Kitayama et al., 1997; May et al., 1997; Motegi et al., 1997). Both of the myosin-II HCs localize to the F-actin ring during mitosis. Myo2 has been shown to be essential for cytokinesis, while Myo3 is not. It is suggested that Myo2 and Myo3 may overlap in function, because function of one myosin-II HC could be replaced by overexpression of the other (Motegi et al., 1997). We previously investigated the phenotype of myo2 Δ myo3 null cells by repressing the expression of myo2* gene in the myo3Δ cells, and observed a synthetic interaction between Myo2 and Myo3 (Motegi et al., 1997). However, in this case, it was unclear whether there was any Myo2 remaining in the cells. In the present study, we analyzed the phenotype of myo2-E1 myo3Δ cells. These cells showed a
severe defect in both cell morphology and F-actin localization at all temperatures tested, whereas the myo2-E1 cells showed a temperature-sensitive phenotype. Thus, it is suggested that Myo3 is involved in cytokinesis under normal conditions. The myo2-E1 myo3Δ cells were incapable of forming the F-actin ring between the segregating nuclei at the restrictive temperature. Simultaneous localization of Myo2 and GFP-Myo3 as the medial ring structure emphasizes the overlap of the function of these myosin-II HCs. Thus, in S. pombe the function of myosin-II HCs is essential for the formation of the F-actin ring.

Cdc4 is an EF-hand protein that is also essential for the formation of the F-actin ring (McCollum et al., 1995), and Cdc4 has been shown to associate with Myo2 probably via the IQ-domain of Myo2 (Naqvi et al., 1999). However, loss of Myo2 function does not lead to complete loss of F-actin ring formation; an aberrant F-actin ring is formed (Kitayama et al., 1997). Thus, we expected that Myo3 would be another target of Cdc4. Immunoprecipitation studies and two-hybrid analysis showed that Myo3 binds to Cdc4 via its IQ-domains. Therefore, Cdc4 appears to be a common light chain that binds to both Myo2 and Myo3. It has been observed that Myo2 forms an improper ring in the myo2Δ cells (Naqvi et al., 1999). We found that Cdc4 staining is not seen in the myo2-E1 myo3Δ cells at the restrictive temperature. These observations suggest that Cdc4 is transported to the division site together with Myo3 in the myo2Δ cells. The myo2-E1 myo3Δ cells at the restrictive temperature showed dispersed F-actin patches, no F-actin cables, and no F-actin ring between the segregating nuclei, like the phenotype of cdc4Δ cells (McCollum et al., 1995). These results suggest that two myosin-II, Myo2-Cdc4 and Myo3-Cdc4 play a similar role in the F-actin organization throughout the cell cycle, including the formation of the F-actin ring during mitosis.
Myo2 may have some interaction with the F-actin cables accumulated in the medial cortex, since these structures colocalize in some regions. Finally, the network is packed into a tight ring structure, in which Myo2 colocalizes with the F-actin ring. Based on several results, which will be discussed below, we classify this assembly process of Myo2 at the division site into two steps: the first is the F-actin-independent Myo2 dot accumulation, and the second is the F-actin-dependent Myo2 assembly from a wide cortical network into a ring.

**F-actin-independent process of Myo2 ring assembly**

The Myo2 dots seemed to be accumulated at the medial cortex before the accumulation of F-actin cables in this region. The Myo2 dot accumulation at the medial cortex was observed in mitotic cdc3<sup>ts</sup>, cdc8<sup>ts</sup> and cdc12<sup>ts</sup> cells. cdc3<sup>+</sup> encodes profilin and cdc8<sup>+</sup> encodes tropomyosin, and in these mutant cells F-actin patches were delocalized as described previously (Balasubramanian et al., 1992, 1994; Chang et al., 1996). Furthermore, no F-actin cable was detected in cdc8<sup>ts</sup> cells as described (Chang et al., 1996; Arai et al., 1998). cdc12<sup>+</sup> encodes a diaphanous family protein, and cdc12<sup>ts</sup> cells showed delocalized F-actin patches during mitosis (Chang et al., 1996, 1997). Thus, proper F-actin organization including F-actin cables may not be necessary for the appearance of Myo2 dots. Furthermore, the Myo2 dots accumulated at the medial cortex even when F-actin was disrupted by Lat-A. These results strongly indicate that F-actin is not essential for the accumulation of the Myo2 dots at the medial cortex of the cell.

A significant amount of Myo2 in the insoluble fraction of the cell lysate was extractable by 1% Triton X-100. This suggests that Myo2 may be interacting with membranous structures. A study on cortices of sea urchin eggs isolated on a glass surface has shown that myosin-II remains in the cleavage furrow after the contractile ring (Fishkind et al., 1997). These studies suggest that the interaction between Myo2 and F-actin occurs during the formation of the F-actin ring. Several myosin-II mutants, cdc4<sup>ts</sup> cells (McCollum et al., 1995; Chang et al., 1996), myo2-E1 cells (Balasubramanian et al., 1998), myo2ΔA cells (Kitayama et al., 1997), myo3Δ cells (Motege et al., 1997), and myo2Δ cells expressing GFP-Myo2ΔATP, which lacks an ATP-binding site (Naqvi et al., 1999), show defects in packing the network of F-actin cables into a tight ring. The defects in myo2-E1 cells or myo2Δ cells expressing GFP-Myo2ΔATP are not due to mislocalization of the mutant Myo2, but may be due to a loss of activity of the mutant Myo2 to organize F-actin cables into a functional F-actin ring. Thus, our observation and the above studies suggest that the interaction between Myo2 and the F-actin cables converts the networks of Myo2 and the F-actin cables into a tight F-actin ring.

By comparison of light and electron micrographs, Verkhovsky and Borisy (1993) have shown that myosin-II spots in the fibroblast lamellipodia consist of minifilament networks of myosin-II that seem to localize independently of the F-actin cytoskeleton. Similar myosin-II minifilaments have been observed in the cleavage furrow of cultured cells (Fishkind et al., 1997). In *Xenopus* eggs myosin-II forms spots in the growing end of the cleavage furrow prior to assembly of F-actin in this region and the myosin-II spots are then converted to fibers along the newly formed F-actin bundles in the contractile ring (T. Noguchi and I. Mabuchi, unpublished). DeBiasio et al. (1996) investigated the dynamics of the fluorescent analogue of myosin-II in living fibroblasts during mitosis. Myosin-II is transported toward the equatorial plane and forms a three-dimensional network composed of spots and fibers during anaphase and telophase. The Myo2 dot of *S. pombe* cells may correspond to the myosin-II spot in vertebrate cells, that is, the Myo2 dot may also be a cluster of minifilaments. This speculation is consistent with our cell fractionation experiment: Myo2 was concentrated in the pellet of the lysate especially from mitotic cells and a significant portion was solubilized at a high ionic strength. This property is that of the myosin-II filament. Therefore, the myosin-II assembly in mitotic *S. pombe* cells may be similar to that in other organisms.

**Function of Cdc4 myosin light chain on the Myo2 ring assembly**

Myo2 staining was not seen in cdc4<sup>ts</sup> cells or cdc4Δ cells, suggesting that Cdc4 is essential for the localization of Myo2.
(Naqvi et al., 1999; this study). Phosphorylation of Cdc4 does not influence the localization of Myo2 (McCollum et al., 1999). It is possible that Cdc4 may interact with other molecules which anchor the tail region of Myo2 to the division site. However, we found that the expression of GFP-Myo2dIQs, which lacks a putative Cdc4-binding site is capable of suppressing the phenotypes of myo2Δ cells, cdc4Δ myo2Δ cells, cdc4Δ myo2Δ cells, and myo2Δ myo3Δ cells. Moreover, GFP-Myo2dIQs assembles into a medial ring in these cells. Naqvi et al. (1999) have also shown that Myo2R770A, which is incapable of associating with Cdc4 in immunoprecipitation studies is able to rescue the myo2Δ cells. These results suggest that localization of Myo2 lacking its IQ-domains is not influenced by Cdc4 (and Myo3). Thus, it is unlikely that unknown targets of Cdc4 regulate the Myo2 localization. D. discoideum cells in which RLC had been disrupted show abnormal localization of myosin-II, and purified RLC null myosin-II shows a defect in filament disassembly in vitro (Chen et al., 1994). In contrast, D. discoideum cells in which the essential light chain had been disrupted show proper localization of myosin-II (Pollenz et al., 1992; Chen et al., 1995). The assembly states of myosin-II play a key modulatory role in controlling myosin-II localization (Egelhoff et al., 1991, 1993; Sabry et al., 1997). Therefore, we consider that Cdc4 may stabilize Myo2 by associating with its IQ-domains in order to maintain Myo2 in a proper state of assembly.

Factors that may be involved in myosin-II assembly in the division site

Recently, the spatial and temporal control of myosin-II assembly has been investigated in S. cerevisiae cells. These cells divide using a contractile ring containing F-actin, MYO1 (myosin-II HC), CYK1 (IQGAP-homologue) and CYK2 (PSTPIP-family protein) (Bi et al., 1998; Lippincott and Li, 1998a;b; Shannon and Li, 1999). MYO1 forms a ring in late G1 or early S phase at the presumptive bud site where septins and chitin have already formed a ring (Bi et al., 1998; Lippincott and Li, 1998). The assembly of CYK1 and F-actin at the mother-bud neck occurs during anaphase (Lippincott and Li, 1998). The assembly of MYO1 is dependent on septins, but independent of F-actin and CYK1 (Bi et al., 1998; Lippincott and Li, 1998). In contrast to S. cerevisiae, Rng2, an IQGAP-homologue in S. pombe, is required for Myo2 assembly (Eng et al., 1998). Septins are also present in S. pombe, but their functions are not clear yet (Longtine et al., 1996). Studies of the function of Rng2 and septins would give an insight into the mechanism of Myo2 assembly.

There may be another approach to study the mechanism of myosin-II assembly at the division site. It is not known what molecules regulate the localization of myosin-II during early mitosis. Myosin-II might be guided to the medial cortex by proteins that are able to recognize the tail region of the heavy chain. Moreover, it is possible that the assembly state of myosin-II is regulated by phosphorylation-dephosphorylation of its heavy chains. Therefore, both phosphorylation-dephosphorylation of the heavy chains and myosin-II-binding proteins would need to be clarified.

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REFERENCES


