Identification of a Second Myosin-II in *Schizosaccharomyces pombe*: Myp2p Is Conditionally Required for Cytokinesis

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As in many eukaryotic cells, fission yeast cytokinesis depends on the assembly of an actin ring. We cloned *myp2*⁺, a myosin-II in *Schizosaccharomyces pombe*, conditionally required for cytokinesis. *myp2*⁺, the second myosin-II identified in *S. pombe*, does not completely overlap in function with *myo2*⁺. The catalytic domain of Myp2p is highly homologous to known myosin-IIs, and phylogenetic analysis places Myp2p in the myosin-II family. The Myp2p sequence contains well-conserved ATP- and actin-binding motifs, as well as two IQ motifs. However, the tail sequence is unusual, since it is predicted to form two long coiled-coils separated by a stretch of sequence containing 19 prolines. Disruption of *myp2*⁺ is not lethal but under nutrient limiting conditions cells lacking *myp2*⁺ function are multiseptated, elongated, and branched, indicative of a defect in cytokinesis. The presence of salt enhances these morphological defects. Additionally, Δ *myp2* cells are cold sensitive in high salt, failing to form colonies at 17°C. Thus, *myp2*⁺ is required under conditions of stress, possibly linking extracellular growth conditions to efficient cytokinesis and cell growth. GFP-Myp2p localizes to a ring in the middle of late mitotic cells, consistent with a role in cytokinesis. Additionally, we constructed double mutants of Δ *myp2* with temperature-sensitive mutant strains defective in cytokinesis. We observed synthetic lethal interactions between Δ *myp2* and three alleles of *cdc11ts*, as well as more modest synthetic interactions with *cdc14ts* and *cdc16ts*, implicating *myp2*⁺ function for efficient cytokinesis under normal conditions.

INTRODUCTION

Many eukaryotic cells divide by a process known as cytokinesis. In the best characterized systems, cell separation occurs via constriction of the plasma membrane mediated by a contractile ring (Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). The contractile ring is composed of antiparallel actin filaments, which encircle the equator just inside the plasma membrane. In a variety of eukaryotic cells, myosin-II is the molecular motor, which provides the force to constrict the contractile ring. However, the molecules which activate myosin-II, thus initiating contraction or the molecules which direct the

site of cleavage furrow formation remain mostly unknown. A model system which is genetically manipulatable would provide insight as to the regulators of cytokinesis.

The fission yeast, *Schizosaccharomyces pombe*, a genetically tractable unicellular organism, has proved to be an excellent model organism by which to study regulation of the cell cycle (Hayles and Nurse, 1989). Fission yeasts divide by medial fission. Actin is localized at the growing ends of the cells during interphase and in early M-phase actin concentrates at the center of the cell, where it forms a ring just inside the plasma membrane (Marks and Hyams, 1985). After completion of nuclear division and segregation, the actin ring constricts, closely followed by formation of a septum, which is deposited from the outside of the cell and

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moves inward. Digestion of the cell wall eventually separates the two daughter cells.

Genetic analysis has identified genes essential for cytokinesis, including *cdc3⁺*, which encodes a profilin, and *cdc8⁺*, which encodes a tropomyosin (Balasubramanian et al., 1992; Balasubramanian et al., 1994). Both proteins bind actin, and in their absence actin is mis-localized throughout the cell cycle (Balasubramanian et al., 1992; Balasubramanian et al., 1994; Chang et al., 1996). A few temperature-sensitive mutants, *cdc4ts*, *cdc12ts*, *cdc15ts*, and *rng2ts*, affect the distribution of actin only during mitosis (Chang et al., 1996). *cdc4⁺* encodes a putative myosin light chain, which localizes to the actin ring during cytokinesis (McCollum et al., 1995), suggesting that a myosin may be involved in cytokinesis in *S. pombe*. During the course of this work, a myosin-II gene (*myo2⁺*) was cloned from the fission yeast, and the product was shown to localize to the actin ring during cytokinesis (Kitayama et al., 1997). Disruption of *myo2⁺* is lethal. When cells are depleted of *myo2⁺*, cells arrest elongated, multiseptated, and occasionally branched. Overexpression of *myo2⁺* is toxic to cell growth and leads to a large number of multinucleated cells with few septa and no apparent actin ring formation. Thus, as in many other eukaryotic cells, myosin-II is required for cytokinesis in fission yeast, demonstrating that *S. pombe* may provide a model system for the study of cytokinesis in general.

We identified a second myosin-II in *S. pombe*, which we named *myp2⁺* for myosin-II of *pombe*. *myp2⁺* may play a more specialized role in cytokinesis than *myo2⁺*. Under conditions of limiting nutrients, the absence of *myp2⁺* function leads to multiseptated cells consistent with a role of *myp2⁺* in cytokinesis. In addition, *myp2⁺* is essential in the presence of 1 M KCl at 17°C. Thus *myo2⁺* cannot replace *myp2⁺* under all conditions. Overexpression of *Myp2p* is toxic, leading to multinucleated cells. In late mitotic cells, GFP-*Myp2p* localizes to either a ring or a dot in the middle of the cell, which precedes the site of septation. Together with the localization, genetic interactions under normal growth conditions between strains lacking *myp2⁺* and other mutants defective in cytokinesis also suggest that *myp2⁺* functions at all times during cytokinesis.

MATERIALS AND METHODS

Phylogenetic Analysis

Myosin sequences were obtained from GenBank (GB),¹ Protein Identification Resource (PIR), and Swiss Prot (SP) databases. Names

and accession numbers are as follows: chicken embryonic skeletal muscle myosin-II, SP: P02565; chicken nonmuscle myosin-IIa, SP: P14105; chicken smooth muscle myosin-II, SP: P10587; chicken skeletal muscle myosin-II, SP: P13538; chicken brush border myosin-I, GB: X58479; chicken myosin-V, GB: Z11718; *Acanthamoeba* myosin-Ib, SP: P19706; *Acanthamoeba* myosin-Ic, SP: P10569; *Acanthamoeba* high-molecular-weight myosin-I, PIR: A23662; *Acanthamoeba* myosin-II, SP: P05659; *Arabidopsis* ATM2, GB: Z34292; *Arabidopsis* Mya1, GB: Z28389; *Arabidopsis* Mya2, GB: Z34294; *Caenorhabditis elegans* myosin-Ia, GB: Z75564; bovine brush border myosin-I, SP: P10568; bovine myosin-X, GB: U55210; *Dictyostelium* myosin-Ic, GB: L35323; *Dictyostelium* myosin-Ia, SP: P22467; *Dictyostelium* myosin-II, SP: P08799; *Dictyostelium* MyoJ, GB: L35322; *Drosophila* myosin-Ia, PIR: S45573; *Drosophila* myosin-Ib, PIR: S45574; *Drosophila* muscle myosin-II, GB: M61229; *Drosophila* 95F, SP: Q01989; *Drosophila* ninaC, SP: P10676; *Entamoeba* myosin-II, GB: L03534; frog myosin-I β , GB: U14549; human myosin-Ic, GB: U14391; mouse myosin-Ia, PIR: L00923; mouse dilute, GB: X57377; pig myosin-VI, PIR: A54818; rat myr1a, PIR: A45439; rat myr3, GB: X74815; rat myr4, PIR: A53933; rat myr5, GB: X77609; *Saccharomyces cerevisiae* MYO1, PIR: S46773; *S. cerevisiae* MYO2, SP: P19524; *S. cerevisiae* MYO4, SP: P32492; *S. pombe* *myo2⁺*, GB: U75357. We used the program Clustal W (Thompson et al., 1994) to create a multiple-sequence alignment of these sequences and to build the tree from the alignment. Bootstrapped distance matrix analysis was performed using Clustal W with 1000 bootstrapping trials. We used the PHYLIP program DRAWTREE to draw the tree from the output of the Clustal W program.

Strains Media and Transformation

S. pombe strains used in this study are listed in Table 1. Yeast culture, methods, media, and genetic manipulations were carried out by standard methods (Moreno et al., 1991). Transformation of *S. pombe* was achieved by electroporation (Moreno et al., 1991) or by a lithium acetate method (Okazaki et al., 1990).

Identification of *myp2⁺*, Cloning, and Sequencing

We used the TblastN algorithm (Altschul et al., 1990) to search the Sanger genome *S. pombe* database (<http://www.sanger.ac.uk/Projects/S.pombe/>) with the chicken skeletal muscle myosin II sequence. We obtained one open reading frame that had a score of 359 and smallest sum probability of 2.6×10^{-215} for $N = 13$. We obtained the nucleotide sequence for the potential myosin-II open reading frame by FTP from the collection of completely sequenced cosmids at the Sanger *S. pombe* genome database (<ftp://ftp.sanger.ac.uk/pub/yeast/sequences/pombe/>).

Using the sequence information from the Sanger genome *S. pombe* database, we designed primers to amplify the *myp2⁺* gene by polymerase chain reaction (PCR). Two PCR products were obtained. One is 2.2 kilobases (kb) and comprises the catalytic domain of *myp2⁺*. The second is the full length *myp2⁺* and is 6.4 kb. These PCR products were subcloned into pBluescript by conventional methods using a *Bam*HI restriction site designed into the 5' PCR primer and a *Sall* restriction site designed into the 3' PCR primer. The PCR products were sequenced by automated sequencing (Applied Biosystems Inc., Foster City, CA). The catalytic domain was intact and had no sequence differences with respect to the sequence obtained from the Sanger database. The original full-length PCR product had several mutations in the 5' end, including a 700-base pair (bp) deletion but was intact from the unique *AccI* site to the 3' end. The region from the *AccI* site to the 3' end was sequenced on both strands, and eight nucleotide differences were found with respect to the sequence obtained from the Sanger database, resulting in three amino acid changes. Because multiple PCR clones contain the same nucleotide differences, we assume that the unfinished sequence provided by the Sanger database was incorrect. To construct an intact *myp2⁺* clone, a *Bam*HI/*AccI* fragment from the catalytic domain was ligated into the full-length construct replacing the incor-

¹ Abbreviations used: GB, GenBank; PIR, Protein Identification Resource; SP, Swiss Prot; PCR, polymerase chain reaction; kb, kilobases; bp, base pair; 5' RACE, 5' rapid amplification of cDNA ends; DAPI, 4'-6-diamidino-2-phenylindole; ORF, open reading frame; ME, malt extract.

Table 1. Strains used in this study

Strain	Genotype
FY435	<i>h⁺ his7-366 leu1-32 ura4-Δ18 ade6-M210</i>
FY436	<i>h⁻ his7-366 leu1-32 ura4-Δ18 ade6-M216</i>
TP5	<i>h⁻ Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18 ade6-M210</i>
TP50	<i>h⁺ Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18 ade6-M216</i>
TP47	<i>h⁻ cdc11-136 his7-366 leu1-32 ura4-Δ18</i>
TP49	<i>h⁻ cdc11-123 his7-366 leu1-32</i>
TP36	<i>h⁻ cdc11-19 his7-366 leu1-32 ura4-Δ18, ade6-M216</i>
TP37	<i>h⁺ cdc14-118 his7-366 leu1-32</i>
TP9	<i>h⁻ cdc8-27 his7-366 leu1-32 ura4-Δ18, ade6-M216</i>
TP32	<i>h⁺ cdc16-116 his7-366 leu1-32 ade6-M210</i>
TP51	<i>h⁺ cdc11-136 Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18 ade6-M216</i>
TP52	<i>h⁻ cdc11-123 Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18</i>
TP53	<i>h⁻ cdc11-19 Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18 ade6-M216</i>
TP54	<i>h⁻ cdc14-118 Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18</i>
TP55	<i>h⁻ cdc16-116 Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18 ade6-M210</i>
TP23	<i>h⁺ cdc8-27 Δmyp2::his7⁺ his7-366 leu1-32 ura4-Δ18 ade6-M216</i>

rect 5' end. This construct is pmyp2BS. The sequence of *myo2⁺* has been deposited into GenBank with accession number AF029788.

To determine the 5' end of *myo2⁺*, we isolated RNA from wild-type cells in midlog growth in yeast extract and supplements media following the manufacturer's recommendations (RNeasy Midi Prep, Qiagen, Chatsworth, CA). We performed 5' rapid amplification of cDNA ends (5' RACE) following the manufacturer's recommendations (Life Technologies, Inc. 5' RACE system). The 5' RACE PCR products were amplified with *Taq* polymerase and subcloned into the TA-cloning vector (Invitrogen, San Diego, CA). The RACE products were sequenced by automated sequencing (ABI).

Disruption of *myo2⁺*

PCR primers were designed based on the sequence obtained from the Sanger database to amplify a 1.4-kb region upstream and overlapping the 5' of *myo2⁺* and a 1-kb region downstream and overlapping the 3' of *myo2⁺* from genomic DNA. The 5' PCR product contains 411 bp of the coding sequence, and the 3' PCR product contains the last 42 bp of coding sequence (Figure 4B). The 5' and 3' PCR products were ligated to an *EcoRI* fragment of pEA2 (Apolinario *et al.*, 1993) containing the *his7⁺* gene, resulting in the linear knockout construct deleting 5858 bp of *myo2⁺* (Figure 4B). The linear fragment was transformed into a *his7-366* wild-type diploid constructed from FY435 and FY436. His⁺ transformants were sporulated on Edinburgh minimal medium, and stably transformed His⁺ haploids were analyzed by colony PCR (C. Troxell, personal communication) to determine the position of integration of the *his7⁺* gene. The forward PCR primer is located within the knockout construct, and the reverse PCR primer is located outside the knockout construct (Figure 4B). Amplification of the disrupted locus results in a PCR product that is 2.5 kb smaller than the wild-type locus.

Complementation and Overexpression

pSGP573 is a GFP-tagging vector that carries the thiamine-repressible *nmt1⁺* promoter (gift of S.G. Pasion). To express *myo2⁺* from the *nmt1⁺* promoter and fused to GFP at the N terminus, a PCR product of the 5' 2.2 kb of *myo2⁺* was amplified from genomic DNA with a primer that anneals immediately downstream of the start methionine. A *NotI* site was engineered into the 5' primer. We verified the sequence of this PCR product and using the unique *AccI* site within

this fragment, we ligated it to the 3' of *myo2⁺*, creating pmyp2BS-1. The *NotI/SalI* fragment from pmyp2BS-1 containing *myo2⁺* without the start methionine was ligated into pSGP573 to construct pGFP-*myo2*. pGFP-*myo2* was transformed into FY436 and TP5 strains. Ura⁺ transformants were selected and streaked on selective EMM ± 1 M KCl with and without thiamine at 36, 32, and 25°C.

Microscopy

For analysis of the phenotype of *Δmyo2*, cells were either removed from plates and resuspended in buffer or visualized directly on plates. We used a 40× long-working-distance bright-field objective (Nikon) to visualize cells on plates. Cells removed from plates and resuspended in buffer were either directly deposited on a glass slide and then visualized by phase contrast microscopy or stained with calcofluor and 4'-6-diamidino-2-phenylindole (DAPI). To stain cells, the cells were spun down and then resuspended in 70% ethanol at room temperature for 10 min. The cells were resuspended in phosphate-buffered saline containing 0.5 μg/ml calcofluor and 0.5 μg/ml DAPI for 10 min at room temperature. The cells were washed twice with phosphate-buffered saline and then resuspended in phosphate-buffered saline and deposited onto a glass microscope slide and covered with a coverslip. Cells were observed with a 100× objective on a Leitz microscope using a filter appropriate for calcofluor and DAPI.

To determine the localization of GFP-Myo2p, cells were grown in culture to exponential phase. Cells were harvested by centrifugation and resuspended in water containing 0.5 μg/ml DAPI and incubated at room temperature for 10 min. Cells were pelleted and then resuspended in water and deposited onto a glass microscope slide, dried down, and covered with 1 mg/ml phenylenediamine in 90% glycerol and a coverslip. Cells were observed with a 63× objective on a Leitz microscope using filters appropriate for DAPI and GFP. All images were photographed with 35-mm slide film, which were digitally scanned and printed using the Macintosh program Adobe Photoshop.

RESULTS

Identification of *myo2⁺* Sequence

A search of the Sanger *S. pombe* genome database using the TblastN algorithm (Altschul *et al.*, 1990) yielded one large open reading frame (ORF) with significant homology to chicken skeletal myosin-II protein sequence. We named this ORF *myo2⁺* for myosin-II of *pombe*. The gene *myo2⁺* encodes a polypeptide of 2104 amino acids and predicted molecular weight of 240,000. The first 824 amino acids are highly homologous to the catalytic domains of myosin heavy chains. To classify Myo2p, we used Clustal W (Thompson *et al.*, 1994), to align the putative catalytic domain of Myo2p with a panel of myosins from several distinct families. From this alignment, we built a phylogenetic tree. The *S. pombe* myosins Myo2p and Myo1p and the *S. cerevisiae* myosin-II MYO1p are related to each other (Figure 1) and as a group join the main branch of myosin-IIs with a bootstrapping value of 55%, which indicates that of the 1000 bootstrapping trials, 55% of the time the yeast myosin-IIs grouped with the main branch and 45% of the time they joined the amoebae myosin-IIs. However, the node of the amoebae myosin-IIs joins the main myosin-II branch 100% of the time and is more distant than the node of

For comparison, the tail of myosin-II from chicken skeletal muscle has no prolines in its tail of 1098 residues. The prediction for chicken skeletal muscle myosin-II indicates that the entire tail forms one coiled-coil (Figure 3), which has been established by

We cloned *myp2*⁺ by amplifying two overlapping sections of *myp2*⁺ from genomic DNA. These segments were each subcloned into pBluescript KS⁺ by conven-

Figure 2. Sequence alignment of *myp2*⁺ with six myosin-II proteins from other organisms. The catalytic domain of *myp2*⁺ was aligned with myosin-II proteins from *S. pombe*, *S. cerevisiae*, amoeba, and chicken using Clustal W (Thompson *et al.*, 1994). From top to bottom: *S. pombe myp2*⁺, *S. pombe myo2*⁺, *S. cerevisiae* myosin-II (MYO1p), *Entamoeba* myosin-II, *Acanthamoeba* myosin-II, and chicken skeletal muscle myosin-II. The sequence accession numbers are given in MATERIALS AND METHODS. The numbers to the left of the amino acid sequence indicate amino acid position. Amino acid identities are shown in white lettering against black, conserved residues are shown in black lettering against gray. The putative ATP-binding motif is underlined. The putative actin-binding site has asterisks underneath. The putative IQ motifs are underlined twice.

tional methods. We verified the sequence of *myp2*⁺. There were eight nucleotide differences from the sequence obtained from the Sanger genome database resulting in three different amino acid residues (see MATERIALS AND METHODS). We obtained a full-length clone (pmyp2BS) by ligating together the amplified overlapping clones using a unique restriction site in the *myp2*⁺ ORF (see MATERIALS AND METHODS).

The 5' end of pmyp2BS has a small ORF of 14 amino acids just upstream and out of frame with the putative start methionine of *myp2*⁺ (Figure 4A). We used 5' RACE PCR (Frohman *et al.*, 1988) to determine the 5' end of *myp2*⁺. The sequenced RACE PCR products all start within the small 14-amino acid ORF (Figure 4A, arrows), suggesting that the first methionine, which is in frame with the *myp2*⁺ ORF, is the start methionine. The RACE PCR also demonstrates that the 5' of *myp2*⁺ is transcribed in wild-type cells at the time the RNA was isolated. In addition, we amplified a 1.4-kb region of the 3' of *myp2*⁺ from a cDNA library. Together with the RACE result, this shows that the *myp2*⁺ RNA is present in wild-type cells during midlog growth in rich media (YES).

Disruption of *myp2*⁺

To examine *myp2*⁺ function, we disrupted *myp2*⁺ in *S. pombe* by replacing 80% of the coding sequence with the *his7*⁺ gene (Figure 4B). Sporulation of the diploid heterozygous for *myp2*⁺ resulted in four viable spores. We verified that *myp2*⁺ was disrupted in His⁺ haploids by amplifying the genomic locus with specific primers (Figure 4B). Amplification of the disrupted locus results in a smaller fragment than of the wild-type locus (Figure 4C). Wild-type and $\Delta myp2$ strains form colonies of similar size at the same rate at all temperatures tested on YES, minimal media (EMM), and malt extract (ME). For example, at 32°C $\Delta myp2$ single colonies form after 2 days on YES. The $\Delta myp2$ strain had no apparent phenotype on YES media. However, on EMM (Figure 5A) or ME (Figure 5B), $\Delta myp2$ colonies are composed of a heterogeneous mix of cells. $\Delta myp2$ cells are often multiseptated, elongated, branched, or slightly swollen. The swollen cells are more prevalent at higher temperatures. Calcofluor staining reveals that the septa are generally abnormal (Figure 5B). In addition, at 17°C $\Delta myp2$ cells have a very pronounced septum, which can be seen by phase contrast microscopy (Figure 5C, arrows). Because multiply septated cells have a nucleus in each compart-

Figure 3. Coiled-coil predictions of myosin-IIs from different organisms. The predictions were generated by the Coils program

ment (Figure 5B), the deletion of *myp2*⁺ probably does not affect the nuclear division cycle. We do not know which components in the EMM or ME trigger the morphological differences in the Δ *myp2* cells. The phenotype observed on EMM and ME is most penetrant when the cells are grown on agar. In liquid EMM culture, Δ *myp2* cells grow at the same rate as wild-type at 32° and 25°C and appear normal except for an elevated number of septated cells. In an exponentially growing EMM liquid culture at 32°C, 10% of wild-type cells are septated, but 20% of Δ *myp2* cells are septated.

The most striking effects of the *myp2*⁺ deletion are seen when the Δ *myp2* cells are grown on plates with 1 M KCl. The morphological phenotype, including multiseptated cells, is more penetrant in YES or EMM with 1 M KCl than on EMM or ME. Δ *myp2* cells also grow more slowly than wild-type on YES-agar + 1 M KCl or EMM-agar + 1 M KCl (Table 2), although growth rates in liquid media with 1 M KCl are the same. By the time wild-type cells have formed colonies on YES-agar + 1 M KCl, Δ *myp2* colonies are smaller and have a large number of elongated, wider cells, with multiple septa (Figure 6, 32°C and 36°C). Lower temperatures enhance the slow-growth phenotype. For example, at 25°C by the time that wild-type forms small colonies, no Δ *myp2* colonies are visible (Figure 6, 25°C). At 17°C wild-type forms colonies after 10 days, but Δ *myp2* forms no colonies even after 21 days (Table 2). The cells at 17°C on 1 M KCl die highly multiseptated and branched, indicative of a defect in cytokinesis. Thus in the presence of 1 M KCl, Δ *myp2* cells are cold sensitive. Together with the Δ *myp2*⁺ phenotype observed with EMM and ME, the phenotype observed in 1 M KCl suggests that *myp2*⁺ is required for growth when the cells are stressed, either by limiting nutrients (EMM or ME) or by salt (1 M KCl).

Complementation of Δ *myp2* Strain

We verified that the cloned *myp2*⁺ could complement the Δ *myp2* disruption phenotype. We constructed a fusion of GFP to the N terminus of Myp2p (see MATERIALS AND METHODS). Expression of GFP-Myp2p is controlled by the *nmt1*⁺ promoter, which allows low levels of expression in the presence of thiamine, and is induced at least 100-fold by removing thiamine from the media (Basi *et al.*, 1993; Forsburg, 1993). The pGFP*myp2* plasmid restores wild-type growth to Δ *myp2* cells on selective EMM with 1 M KCl

Figure 3 (cont). (Lupas *et al.*, 1991) for *S. pombe* Myp2p, *S. cerevisiae* myosin-II (MYO1p), *S. pombe* Myo2p, and chicken skeletal muscle myosin-II. x-axis, amino acid position. y-axis, probability of forming a coiled-coil. A window of 28 amino acids was used to generate the profiles shown.

Figure 4. Cloning 5' of *myp2*⁺ and construction of the *myp2*-disrupted strain. (A) The DNA sequence for the 5' region of *myp2*⁺ in the pmyp2BS construct is shown along with the deduced amino acid sequence. The arrows indicate the position of the beginning of four separate 5' RACE PCR products that were sequenced. The putative start codon and methionine are highlighted with white lettering against black. (B) Schematic representation of the genomic locus of *myp2*⁺. The shaded arrow represents the open reading frame of 6312 bp. The primers used to amplify the 5' and 3' regions of *myp2*⁺ that were used to generate the knockout construct are represented above the locus. The restriction sites used to ligate together the knockout construct are indicated at the ends of the primers. B, *Bam*HI; E, *Eco*RI; X, *Xho*I. The primers used to amplify the genomic locus from His⁺ haploids generated from the diploid transformed with the knockout construct are indicated below the locus. (C) Amplification of the genomic locus by colony PCR from His⁺ haploids. PCR products were separated on an 0.8% agarose gel. Lane 1 represents a homologous recombination event resulting in disruption of the *myp2*⁺ locus by *his7*⁺. The disrupted locus is 2.5 kb smaller than the wild-type locus. Lane 2 represents a heterologous recombination event in which *his7*⁺ has been integrated elsewhere in the genome thus leaving the *myp2*⁺ locus intact. Lane 3 is the molecular weight marker with sizes in kilobases indicated to the right.

at 32°C in the presence of thiamine (Figure 7A). The $\Delta myp2$ transformants have normal morphology, and the colony size is the same as wild-type. Thus, GFP-Myp2p is functional. Expression of GFP-Myp2p was verified by microscopy (see below). Additionally, low levels of expression of GFP-Myp2p have no effect on colony formation of wild-type cells (Figure 7A).

Overexpression of GFP-Myp2p

Overexpression of GFP-Myp2p is accomplished by removing thiamine from the medium. On EMM plates without 1 M KCl, both wild-type and $\Delta myp2$ cells are able only to form pinprick colonies in the absence of thiamine (Figure 7B). The cells present in the pinprick colonies are multinucleated and highly elongated. Like the phenotype of $\Delta myp2$ cells, overexpression of GFP-Myp2p results in a defect in cytokinesis. However, the two phenotypes differ in that overexpressing GFP-Myp2p results in longer cells with very few septa and no branches. Thus overexpression of functional Myp2p is toxic to cell growth possibly by inhibition of cytokinesis and septation.

Localization of GFP-Myp2p

To observe the subcellular localization of GFP-Myp2p, we grew wild-type cells and $\Delta myp2$ cells transformed with pGFPmyp2 in selective EMM culture with thiamine to exponential phase. Cells in late mitosis con-

tain either a ring (Figure 8, top, arrow) or a bright spot (Figure 8, middle, arrow) of GFP-Myp2p in the middle of the cell. The GFP-Myp2p ring is consistent with actin ring localization during cytokinesis and precedes the site of septation. The spot may represent the localization either before ring formation or after contraction of the ring. In interphase, GFP-Myp2p mostly appears concentrated in a dot in the cytoplasm, usually near the nucleus (Figure 8, bottom, arrow). The distribution of GFP-Myp2p was similar in wild-type and $\Delta myp2$ cells. The intensity of GFP fluorescence is variable between cells, which may reflect variable expression from the episome. However, our complementation results show that sufficient functional GFP-Myp2p is expressed to complement the $\Delta myp2$ phenotype.

Genetic Interactions of $\Delta myp2$ with Cytokinesis Temperature-sensitive Mutants

From the phenotypic analysis of the $\Delta myp2$ strain, we know that *myp2*⁺ function is important for cytokinesis under specific growth conditions. To determine whether the loss of *myp2*⁺ function abrogates cytokinesis under normal growth conditions in YES media, we investigated genetic interactions between $\Delta myp2$ and known regulators of cytokinesis. Double mutant strains were constructed between $\Delta myp2$ and strains with temperature-sensitive mutations in *cdc3*⁺, *cdc4*⁺,

Figure 5. Phenotype of $\Delta myp2$ strain. (A) Wild-type and $\Delta myp2$ strains on EMM-agar grown at 36°C (top), 32°C (middle), and 25°C (bottom). Cells were taken from YES plates and streaked onto EMM plates. Cells were visualized using a 40 $\times$ long-working-distance bright-field objective and photographed directly off the plate. Both strains form groups of cells that will eventually form colonies of roughly the same size. Many of $\Delta myp2$ cells have multiple septa, and a few are branched (arrow). $\Delta myp2$ cells are slightly wider than wild-type and are occasionally swollen (arrowheads) at the higher temperatures. (B) Fluorescence micrographs of wild-type and $\Delta myp2$ cells grown on ME agar, removed from the plate, and stained with DAPI and calcofluor. (C) Phase contrast micrographs of wild-type and $\Delta myp2$ cells grown on EMM agar at 17°C smeared on a microscope slide. $\Delta myp2$ cells have very pronounced and misshapen septa (arrows).

Table 2. Rate of colony formation in YES^a + 1 M KCl

Temperature (°C)	Wild-type	$\Delta myp2$
36	3d	5 d
32	3d	7 d
25	6d	12 d
17	10d	No colonies

^a Colony formation in EMM + 1 M KCl is the same for each strain at each temperature.

cdc8⁺, *cdc11*⁺, *cdc14*⁺, and *cdc16*⁺. *cdc3*⁺ encodes a profilin (Balasubramanian *et al.*, 1994), *cdc8*⁺ encodes a tropomyosin (Balasubramanian *et al.*, 1992), and *cdc4*⁺ encodes a putative myosin light chain (McCollum *et al.*, 1995). These three genes are localized at the contractile ring during cytokinesis, and the temperature-sensitive alleles are characterized as late septation mutants with defects in the organization of the septum and the actin contractile ring (Balasubramanian *et al.*, 1992; Balasubramanian *et al.*, 1994; McCollum *et al.*, 1995; Chang *et al.*, 1996). *cdc11ts* and *cdc14ts* are early septation mutants given that they are unable to form a septum and arrest with multiple nuclei. *cdc11*⁺ and *cdc14*⁺ are cloned and encode novel proteins (Fankhauser and Simanis, 1994b). *cdc16*⁺ is homologous to *S. cerevisiae* BUB2; it functions in the mitotic checkpoint as well as in regulation of septum formation (Fankhauser *et al.*, 1993). We did not observe any interactions between the late septation mutants (*cdc3ts*, *cdc4ts*, and *cdc8ts*) and $\Delta myp2$. However, we did observe synthetic interactions between $\Delta myp2$ and the early septation mutants (*cdc11ts* and *cdc14ts*) and *cdc16ts*.

All three alleles of *cdc11ts* we tested (*cdc11-19*, *cdc11-123*, and *cdc11-136*) were synthetically lethal with $\Delta myp2$. $\Delta myp2$ *cdc11-123* can only form pinprick colonies at 32°C, while both parent strains grow like wild-type (Figure 9, top). We observed a similar phenotype for $\Delta myp2$ *cdc11-19*. *cdc11-136* can form pinprick colonies at 32°C, but $\Delta myp2$ *cdc11-136* cannot form colonies at all (Figure 9, top). For $\Delta myp2$ *cdc14ts* and $\Delta myp2$ *cdc16ts*, there was a modest reduction in colony size (Figure 9, bottom) and the double mutants were darker pink on phloxin-B than any of the single mutants. Given the strong interaction of $\Delta myp2$ with *cdc11ts* and the modest interaction with *cdc14ts* and *cdc16ts*, we conclude that *myp2*⁺ functions in normal growth conditions and contributes to septation.

Figure 5 (cont). $\Delta myp2$ cells grown on ME agar, removed from the plate, and stained with DAPI and calcofluor. (C) Phase contrast micrographs of wild-type and $\Delta myp2$ cells grown on EMM agar at 17°C smeared on a microscope slide. $\Delta myp2$ cells have very pronounced and misshapen septa (arrows).

Figure 6. Phenotype of $\Delta myp2$ cells grown on YES + 1 M KCl at 36°C (top), 32°C (middle), and 25°C (bottom). Cells were streaked onto YES + 1 M KCl plates from YES plates. Cells were visualized using a 40 $\times$ long-working-distance bright-field objective and photographed directly off the plate. $\Delta myp2$ cells eventually form colonies at all three temperatures but are slower than wild-type, as apparent from the smaller colonies at 36°C and 32°C.

DISCUSSION

We have identified and characterized a second myosin-II from fission yeast, *myo2⁺*. Phylogenetic analysis is useful to categorize myosins into subfamilies based on the sequences of the catalytic domain (Mooseker and Cheney, 1995). Having no assay for *myo2⁺* function, we used phylogenetic analysis to determine its subfamily. Myp2p groups with the yeast myosin-II's which join the main branch of myosin-II's near the node of the amoebae myosin-II's. We are confident that Myp2p is a type II myosin. The catalytic domain contains a well-conserved ATP-binding motif, an actin-binding motif, and like most myosin-II's two IQ motifs that mediate light chain binding. The first IQ motif is relatively well-

Figure 7. Complementation by pGFPmyp2 and overexpression of pGFPmyp2. Ura⁺ transformants of strains carrying the indicated plasmid (pSGP573 or pGFPmyp2) were streaked to (A) EMM-Ura + 1 M KCl + thiamine (B1) (this plate shows colonies after 4 days at 32°C) and to (B) EMM-Ura (this plate shows colonies after 3 days at 32°C).

conserved except that it does not contain the first isoleucine and the second glutamine residues. Like most myosin-II's, the second IQ motif of Myp2p is degenerate. The second IQ motif, however, does contain the invariant arginine, involved in light chain binding, in the middle of the motif (IQXXRGXXR), which Myo2p, another *S. pombe* myosin-II, does not. Further biochemical and genetic analyses are required to determine the light chain content for both *S. pombe* myosin-II's.

Figure 8. Localization of GFP-Myp2p in $\Delta mvp2$ cells. $\Delta mvp2$ cells were grown in liquid with thiamine and harvested during exponential growth. Cells were stained with DAPI and observed immediately after staining. GFP-Myp2p localizes to a ring in the middle of late mitotic cells (arrow, top). Some late mitotic cells only show a bright spot in the middle of the cell (arrow, middle). Cells in interphase, show GFP-Myp2p localizing to a bright dot near the nucleus (arrow, bottom).

Although the catalytic domain places Myp2p as a myosin-II, the Myp2p tail differs from most other myosin-II, having a long sequence containing 17 prolines that divides two regions predicted to form coiled-coils. We used the most stringent conditions for the Coils prediction algorithm by choosing a window of 28 amino acids to ascertain the probability of forming a coiled coil (Lupas *et al.*, 1991). The Paircoil algorithm (Berger *et al.*, 1995) predicted a similar coiled-coil profile. *S. cerevisiae* myosin-II, MYO1p, has a nonhelical gap containing 6 prolines in a similar position in the tail. MYO1, like *mvp2*⁺, is not essential and the absence of MYO1 leads to long chains of budded cells (Watts *et al.*, 1987). *S. pombe* Myo2p contains 9 prolines and several of these are restricted to an analogous

region as MYO1p and Myp2p. However, the Myo2p tail is shorter and does not appear to contain the second coiled-coil-rod domain. Biochemical studies of the tails from these myosins will be necessary to determine the coiled-coil content of each and whether the differences in the tails may have some relevance to the function of each myosin.

Insight as to the functions of these type II myosins, *mvp2*⁺ and *myo2*⁺, in *S. pombe* can be obtained from studying the phenotypes of strains disrupted for each myosin. Disruption of *mvp2*⁺ is conditional, whereas disruption of *myo2*⁺ is lethal. $\Delta mvp2$ cells exhibit a phenotype including multiseptated cells, which is consistent with a decreased efficiency of cytokinesis under nutrient limiting conditions (EMM or ME). If the $\Delta mvp2$ cells are given an additional stress by adding 1 M KCl to the media, then in addition to the multiseptated phenotype, the growth rate is impaired, and at 17°C the $\Delta mvp2$ cells die multiseptated. This suggests that *mvp2*⁺ is activated under conditions of stress. In response to high salt, several MAP kinases are activated in *S. pombe* (Shiozaki and Russell, 1995; Millar *et al.*, 1995; Degols *et al.*, 1996; Kato *et al.*, 1996; Zaitsevskaya-Carter and Cooper, 1997). The downstream targets of these kinases are not known. Interestingly, the phenotype of the disruption of one of these kinases, *spm1*⁺, is similar to $\Delta mvp2$: $\Delta spm1$ cells are viable and wild-type in appearance in rich media but suffer morphological defects similar to the $\Delta mvp2$ phenotype on minimal media. Similarly, this phenotype is enhanced under high-salt conditions (Zaitsevskaya-Carter and Cooper, 1997). One intriguing possibility is that *mvp2*⁺ activity under these conditions may be affected by the *spm1*⁺ pathway.

Low level episomal expression of GFP-Myp2p complements the disruption phenotype of $\Delta mvp2$. In late mitotic (binucleate) cells, GFP-Myp2p localizes to a ring at the center of the cell, similar to the localization of the actin ring and GFP-myosin during cytokinesis. In interphase cells, GFP-Myp2p was apparent as a dot near the nucleus, much like GFP-myosin (Kitayama *et al.*, 1997).

Overexpression of GFP-Myp2p causes a defect in cytokinesis that differs clearly from the defect in $\Delta mvp2$ cells. Understanding the mechanism of the defect caused by overexpression will require more work, but one possibility is that the excess Myp2p may compete with Myo2p for a limiting pool of shared light chains. Consistent with this, overexpression of Myo2p causes a very similar phenotype as overexpression of Myp2p (Kitayama *et al.*, 1997).

Regulation of cytokinesis in *S. pombe* requires the product of the *cdc15*⁺ gene, which is thought to activate *cdc16*⁺ (Marks *et al.*, 1992). The *cdc16ts* mutant has a unique phenotype, in which cells arrest as binucleates with multiple septa. This suggests that *cdc16*⁺ is involved in coupling the nuclear cycle to septation

Figure 9. Genetic interactions observed with $\Delta myp2$ strain and several temperature-sensitive mutant strains that affect cytokinesis. Cells of the indicated strains were streaked onto YES + phloxin-B plates at the indicated temperatures and then scanned in once the wild-type colonies have formed. In all cases, the double mutants were darker pink than the single mutants indicating that the double mutants are sicker.

(Fankhauser *et al.*, 1993). Mutants defective in the early stage of septum formation, $cdc7ts$ and $cdc14ts$, are synthetically lethal with each other and with $cdc16ts$. $cdc7^+$ encodes a protein kinase and $cdc14^+$ encodes a novel protein (Fankhauser and Simanis, 1994a,b). Several alleles of $cdc11ts$ [encoding another novel protein (Fankhauser and Simanis, 1994b)] are also synthetically lethal with $cdc16ts$. It has been suggested based on these observations that these gene products interact with one another to initiate the formation of the septum and to couple this process to the cell cycle (Marks *et al.*, 1992). Septum formation occurs after the assembly of an actin ring. Activation of a myosin motor may provide the force of contraction. Cdc4p, an apparent myosin light chain, associates with the actin ring, as does Myp2p and Myo2p (Kitayama *et al.*, 1997; McCollum *et al.*, 1995).

The localization of Myp2p during late mitosis, together with the genetic interactions of $\Delta myp2$ with $cdc11ts$ and to a lesser extent $cdc14ts$ and $cdc16ts$, suggests that Myp2p acts during normal cytokinesis, as well as under conditions of stress. Interestingly, $\Delta myp2$ does not interact with any of the late septation mutants ($cdc3ts$, $cdc4ts$, and $cdc8ts$) that affect actin ring formation. Possibly, the interactions observed with the early septation mutant $cdc11ts$ may provide a link between the actin-mediated events at cytokinesis and

initiation of septation, since Myp2p localizes to the same area as actin in late mitotic cells.

Why does fission yeast need two myosin-II genes? In both *S. pombe* and *S. cerevisiae*, there is precedent for multiple genes encoding proteins with overlapping function that may allow the cell to fine tune its response to different growth conditions. For example, in *S. pombe* there are two α -tubulin genes (Hiraoka *et al.*, 1984; Toda *et al.*, 1984). Both are expressed and present in the α -tubulin pool, although possibly because of levels of expression, only one is essential (Adachi *et al.*, 1986). We note that Myo2p and Myp2p are not completely redundant, but they may overlap in function. $\Delta myo2$ spores die with a multiseptate phenotype. This could reflect that a limiting amount of Myo2p is packaged from the parent diploid. Alternatively, it could reflect a limited ability of Myp2p to function in lieu of Myo2p during germination of the $\Delta myo2$ spore. In contrast, deletion of $myo2^+$ causes defects in cytokinesis that are specific to media and cell stress. Thus, Myo2p is not sufficient under these conditions for normal cell growth in the absence of Myp2p. Interestingly, the lethality of $\Delta myp2$ cells at 17°C in 1 M KCl may reflect that the catalytic activity of Myo2p is highly sensitive to the growth temperature as is known for muscle myosin-II's (White and Taylor, 1976). Perhaps these myosins are used selectively to

regulate cytokinesis under different growth conditions. This could occur via differential expression, association with different light chains, or responding to unique signaling pathways in the cell. Further characterization of *myp2*⁺, including analysis of *myp2*⁺ and *myo2*⁺ double mutants, will help us to determine how the second myosin-II functions in fission yeast cytokinesis.

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