The F-actin-based motor myosin II plays a key role in cell division in a variety of eukaryotes (16). Forces produced on interaction of F-actin and myosin II have been proposed to result in the physical severing of one cell into two. Type II myosins are hexameric proteins composed of two heavy chains, two essential light chains, and two regulatory light chains. The heavy chains dimerize on the basis of the coiled-coil sequences in their C termini, and these assembled heavy chains, two essential light chains, and two regulatory light chains. The heavy chains dimerize on the basis of the coiled-coil sequences in their C termini, and these assembled heavy chains also associate to form thick filaments (11). During cytokinesis, myosin II is detected at the cell division site as a component of the actomyosin ring (13, 14, 18). The mechanism of assembly of the myosin II complex and its localization to the cell division site have generated substantial interest.

In recent years, the fission yeast Schizosaccharomyces pombe has emerged as a powerful model organism with which to study cytokinesis, due to its well characterized cell cycle and the availability of mutants defective at various steps in cytokinesis. Importantly, fission yeast cells divide through the use of an actomyosin ring, composed of over 20 proteins, including two type II myosins, Myo2p and Myp2p (12). Myo2p is essential for cytokinesis and cell viability; it associates with two light chains, Cdc4p and Rlc1p (19, 23, 27). Myo2p is detected at the division site during cytokinesis in fission yeast cells (18, 22).

A growing body of evidence from a diverse set of organisms ranging from unicellular to multicellular eukaryotes has implicated the involvement of proteins containing UCS domains in myosin structure and/or function (40). The phenotypes observed on disruption of genes encoding UCS domain proteins indicate abnormalities in specific myosin-associated processes. The UCS domain-containing protein in S. pombe, Rng3p, has previously been shown to be essential for formation of the cytokinetic actomyosin ring, with several lines of evidence indicating an involvement of Rng3p in modulation of Myo2p function (39).

An additional player was introduced when Barral et al., in a landmark study (7), showed that the Caenorhabditis elegans UCS domain protein (Unc45p), which is essential for myosin assembly into thick filaments (6), bound the chaperone protein Hsp90. Hsp90-like proteins have been identified from numerous eukaryotes and aid in the folding, maintenance, and regulation of a diverse set of proteins including cochaperones, kinases, and transcription factors (30). Recent work by Srikakulam and Winkelmann (34) demonstrates that nascent myosin filaments in differentiating muscle cells consist of myosin molecules with unfolded motor domains in a complex with Hsp90 and Hsc70 chaperone proteins.

In this study, we have characterized the in vivo role of Swo1p (the fission yeast Hsp90 homolog) in myosin II assembly and/or function in S. pombe. We show that Swo1p is important for myosin II function in fission yeast and that it cooperates with Rng3p to maintain Myo2p in a functionally stable form.
standard techniques (25). Vegetative cells were grown in YES medium (25), myo2-E1 mrg3-65 and myo2-E2 swo1-GFP strains were grown to early log phase in YES medium supplemented with 1 M Sorbitol, after which the cells were given two washes in YES medium and grown in YES medium for 4 h at 36°C. Genetic crosses were performed by mixing appropriate strains of opposite mating type on YPD plates, and recombinant strains were selected by tetrad dissection carried out on an LSM micromanipulator (Singer Instruments UK). Double mutants were typically isolated from nonparental diploids (NPD) tetrads. Geladnamycin (Sigma, St. Louis, Mo.) was used at 2 μg/ml in dimethyl sulfoxide (DMSO). Sorbitol was used at a final concentration of 1.2 M. Fission yeast transformations were done using the lithium acetate method (26).

**Fluorescence and time-lapse microscopy.** *S. pombe* cells were fixed for 10 min with 3.7% formaldehyde for staining of nuclei with 4,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml (5). Alexa 488-conjugated phalloidin at 100 U/ml, and aniline blue at 0.5 μg/ml. DAPI and aniline blue were dissolved in antifade (1 mg/ml). DAPI and aniline blue were dissolved in antifade (1 mg/ml). DAPI and aniline blue were dissolved in antifade (1 mg/ml). DAPI and aniline blue were dissolved in antifade (1 mg/ml). DAPI and aniline blue were dissolved in antifade (1 mg/ml). DAPI and aniline blue were dissolved in antifade (1 mg/ml). DAPI and aniline blue were dissolved in antifade (1 mg/ml).

**Protein extraction, immunoblotting, and immunoprecipitation.** Protein extracts were prepared from log-phase cells by bead beating in NP-40 buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 6 mM NaH$_2$PO$_4$, 4 mM NH$_4$PO$_4$, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, supplemented with protease inhibitor (Complete, EDTA-free; Roche Diagnostics)). For immunoblotting, cells were cultured in YES medium supplemented with 1.2 M sorbitol to an optical density at 595 nm of 0.2, washed twice with YES medium, and grown in YES medium at 36°C for 5 h. Cell extracts were clarified by centrifugation at 16,100 × g for 10 min at 4°C. Equal amounts of total proteins were loaded on sodium dodecyl sulfate (SDS) 0-12% polyacrylamide gels. Myo2p and Arp3p (used as control for normalization) were detected using affinity-purified polyclonal antibodies as previously described (39). Immunoblots were quantitated by densitometry (Bio-rad). For each immunoprecipitation, 3 to

**Construction of Swolp-GFP.** Plasmid pCDL927 was created to generate a strain that expressed a Swolp-GFP carboxy-terminal fusion protein from its genomic locus. To make pCDL927, the first 1 kb of the swo1-GFP gene was amplified by PCR using primers bearing the Kpn1 (5′-CGCGCGGTACCGGCCGCGG) TTTCTATTCCGAGCG3′) and Sma1 (5′-CCCGCCCGGCGGATCCCGCTCCATCTCTGTC3′) sites. The 5′ primer with the Kpn1 site carried an in-frame stop codon to prevent translation of the untagged copy. The 1-kb Kpn1-swo1-Sma1 fragment was directionally cloned upstream and fused in frame in the GFP gene of the pHK210 GFP plasmid (39). Plasmid pCDL927 was linearized with EcoRI and introduced into the wild-type strain MBY192 (ura4-D18 leu1-32 ura4-D18 h+), creating strain MYB2440 (swo1-GFP::ura4-D18 leu1-32 ura4-D18 h+). Putative integrants selected on plates lacking uracil were subjected to PCR analysis and nucleotide sequence determination to confirm that the desired integration had occurred. Genetic crosses between the individual cytokinesis mutants and MYB2440 were used to create all cytokinesis mutant strains that express the Swolp-GFP fusion.

**Two-hybrid system.** A two-hybrid interaction assay using a 5-bromo-4-chloro-3-indolyl-β-d-galactosidase (X-Gal)-based two-hybrid system was carried out using the Matchmaker two-hybrid system (Clontech Laboratories Inc.). The full-length *S. pombe* mrg3 gene was amplified from genomic DNA by PCR with primer 5′-CCGGTGACATGGAGATGACCCACGAGCTTTCCTC as the 5′ primer and CGCGATCCCTTCTGCTCTCTGGA as the 3′ primer and cloned into the NcoI-BamHI sites of the Gal4BD (pAS2-1) and Gal4AD (pACT2) vectors to create pCDL839 and pCDL841, respectively. The S. pombe mrg3 gene corresponding to amino acids 1 to 836 (which includes the head and the two IQ domains of Myo2p) was similarly cloned, using CATGCCCATGGAGATGACCAAGGAATATCTATCAAATAATAAG as the 5′ primer and TCCGCCGGTAGATTGAAAATAATCACTGTGTT as the 3′ primer, into the NcoI-BamHI sites of Gal4BD (pAS2-1) and Gal4AD (pACT2) vectors to create pCDL839 and pCDL841, respectively. The two types of hybrid plasmids (which carry the yeast TRP1 and LEU2 marker genes) were then cotransformed into the yeast strain Y190 (which is auxotrophic for tryptophan and leucine and has lacZΔ and his3Δ marker genes, each under the independent control of two different GAL4 promoters) using the lithium acetate method of transformation (15). The transformants were first replica plated onto medium lacking leucine and tryptophan to select for those that contained both plasmids. Primary transformants were replica plated onto medium containing 35 mM 3-aminoatrolizin and lacking histidine as well as tryptophan and leucine to check for expression of the HIS3 reporter gene, indicative of a protein-protein interaction. This interaction was further confirmed by determining the function of the lacZ reporter in His+ Trp+ Leu+ transformants by assaying for β-galactosidase activity using a colony-lift filter assay as described in the Clontech manual.

**Protein extraction, immunoblotting, and immunoprecipitation.** Protein extracts were prepared in NP-40 buffer as described previously (37). Briefly, total-cell extracts were prepared from log-phase cells by bead beating in NP-40 buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 6 mM NaH$_2$PO$_4$, 4 mM NH$_4$PO$_4$, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, supplemented with protease inhibitor (Complete, EDTA-free; Roche Diagnostics)). For immunoblotting, cells were cultured in YES medium supplemented with 1.2 M sorbitol to an optical density at 595 nm of 0.2, washed twice with YES medium, and grown in YES medium at 36°C for 5 h. Cell extracts were clarified by centrifugation at 16,100 × g for 10 min at 4°C. Equal amounts of total proteins were loaded on sodium dodecyl sulfate (SDS) 0-12% polyacrylamide gels. Myo2p and Arp3p (used as control for normalization) were detected using affinity-purified polyclonal antibodies as previously described (39). Immunoblots were quantitated by densitometry (Bio-rad). For each immunoprecipitation, 3 to

**TABLE 1. S. pombe strains used in this study**

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<td>Laboratory collection</td>
</tr>
<tr>
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<td>This study</td>
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4 mg of soluble protein was incubated with 5 μl of anti-Myo2p, anti-GFP (Molecular Probes), or anti-HA (12CA5; Sigma) antibodies for 1 h at 4°C. Then 100 μl of preswollen Sepharose-protein A beads (Amersham Biosciences) was added to the antigen-antibody immunocomplex, and the mixture was incubated for 45 min at 4°C. The beads were washed six times with 1 ml of NP-40 buffer with 10-min intervals of incubation on the rotor. After the final wash, the beads were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) gel loading buffer and heated at 95°C for 5 min.

Proteins were separated on SDS-8% polyacrylamide gels and transferred to a polyvinylidene difluoride sequencing membrane (Millipore Corp., Bedford, Mass.). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline-Tween 20. Primary anti-GFP (Molecular Probes) and 12CA5 (Sigma) antibodies were used at 1:2,000 dilutions whereas anti-Myo2p was used at a 1:400 dilution. Peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin G (Sigma) were used at 1:5,000 dilutions, and the enhanced chemiluminescent signal was detected using the ECL1 kit (Amersham) as specified by the manufacturer.

RESULTS

Characterization of the cytokinetic phenotype in swo1-w1 cells. Two temperature-sensitive mutant alleles of the fission yeast Hsp90 chaperone-encoding gene, swo1, have been previously isolated (1, 26). While these studies mainly characterized genetic interactions between the two mutants (swo1-w1 and swo1-26) and the Cdc2 mitotic machinery, it was also observed that the swo1-w1 mutant developed cytokinetic defects when incubated at the restrictive temperature (26). To further characterize the cytokinetic defect in the swo1-w1 mutant, we cultured cells under permissive temperature conditions and then shifted the cultures to the restrictive temperature of 36°C. The cells were fixed and stained with DAPI and aniline blue to visualize the nuclei and septa, respectively. Interestingly, whereas wild-type cells contained approximately 22% binucleate cells and approximately 78% cells with a single nucleus, dramatic cytokinesis defects were detected in the swo1-w1 mutant at the restrictive temperature (Fig. 1A). Only 12% of heat-arrested swo1-w1 cells contained a single nucleus; approximately 42% of the cells contained four or more nuclei, whereas the rest contained two nuclei (Fig. 1B). The septa in swo1-w1 cells were aberrant in appearance (Fig. 1A). Generally, aberrant septation results from assembly of improper actomyosin rings, since constriction of the actomyosin ring is required for and precedes septum assembly.

The swo1-w1 mutation leads to defects in actomyosin ring assembly. To assess the status of the actomyosin ring in heat-arrested swo1-w1 cells, we looked at three components of the actomyosin ring in these cells, namely, F-actin and the heavy and regulatory light chains of type II myosin (Myo2p and Rlc1p, respectively). Wild-type cells and swo1-w1 mutant cells were grown at the permissive temperature of 25°C, shifted to the restrictive temperature of 37°C for 7 h, fixed, and stained with DAPI and Alexa 488-conjugated phalloidin to visualize DNA and F-actin structures. The cortical F-actin distribution in interphase swo1-w1 mutant cells was similar to that of control cells; F-actin patches were concentrated at both cell ends, and F-actin cables were oriented along the long axis of the cell (Fig. 2A). Mitotic mutant cells, however, lacked a well-defined ring of F-actin and had arrays of loose medial actin cables instead (Fig. 2A). In addition to F-actin, Myo2p and Rlc1p were improperly assembled at the restrictive temperature in swo1-w1 mutants undergoing mitosis, as determined by the presence of mitotic spindles (Fig. 2B and C). These studies utilizing the cytokinesis-defective mutant, swo1-w1, established that Swo1p is important for actomyosin ring assembly and that the defect in actomyosin ring function presumably leads to the assembly of improper division septa.

The swo1-w1 mutant shows a genetic interaction with myo2-E1 and rng3-65 alleles. The improper septa observed in swo1-w1 cells resembled those observed in cells carrying null mutations in the genes encoding myosin II heavy chain, Myo2p (4), and the UCS domain protein, Rng3p (39), suggesting that Hsp90 function might be important for assembly and/or function of myosin II. Detailed in vitro biochemical studies have shown that the C. elegans myosin II head domain forms a stoichiometric complex with Hsp90 and Unc45p, a C. elegans protein related to S. pombe Rng3p, indicative of a substrate-chaperone-cochaperone relationship among these three proteins (7). To further analyze if Swo1p was similarly associated with myosin II in S. pombe, we assessed genetic interactions among swo1-w1, rng3-65, and three different mutant alleles of myosin II, myo2-E1, myo2-S1, and myo2-S2. It is noteworthy that previous work has shown that rng3-65 displayed a synthetic lethal genetic interaction with the myo2-E1 alleles but not with the myo2-S1 or myo2-S2 alleles (39). Interestingly, we found that swo1-w1 also displayed a strong synthetic lethal interaction with myo2-E1 (Fig. 3A). Double mutants with mutations of the genotype myo2-E1 swo1-w1 were inviable at all growth temperatures, whereas the two parental strains were compromised for cell viability only at higher temperatures (data not shown). Microscopic examination revealed that the double mutants had undergone spore germination and died as elongated single cells (Fig. 3B). To further characterize the phenotype of
the myo2-E1 swo1-w1 cells, we rescued the double mutants by tetrad dissection on plates containing sorbitol (data not shown). Wild-type cells as well as mutants with mutations of the genotypes myo2-E1, swo1-w1, and myo2-E1 swo1-w1 were grown at 24°C in medium containing sorbitol and subsequently shifted to medium lacking sorbitol. The cells were then fixed and stained with DAPI and aniline blue. Interestingly, we found that the double mutants were specifically defective in FIG. 2. Swo1p function is essential for assembly of a normal actomyosin ring. (A) Wild-type (WT) and swo1-w1 cells were grown at 25°C to exponential growth phase and shifted to 37°C for 7 hrs, fixed, and stained with DAPI and Alexa 488-conjugated phalloidin to visualize chromosomes (blue) and F-actin (green). (B) Similarly treated cells of the two genotype were processed for indirect immunofluorescence with anti-Myo2p and anti-tubulin to visualize Myo2p and microtubule structures, respectively. (C) swo1-w1 cells and wild-type cells expressing Rlc1-GFP were grown at 25°C to exponential growth phase and shifted to 37°C for 7 h, fixed, stained with DAPI to visualize chromosomes (blue), with anti-GFP to visualize Rlc1p (green), and with anti-tubulin to visualize microtubule structure (red). The figures shown are enlarged, merged images from a stack of 16 confocal z-sections of 0.4 μm that have been reconstructed and processed using Volocity Visualization (Improvision) software.
FIG. 3. The swo1-w1 mutant shows a synthetic lethal interaction with both myo2-E1 and rng3-65 mutant strains. (A) The swo1-w1 mutant was crossed with cells of the myo2-E1 genotype, and resulting tetrad were dissected and grown at 24°C. Four spores from each tetrad are aligned vertically; seven tetrads are shown. P, parental ditype (all spores viable and showing a temperature-sensitive phenotype); N, nonparental ditype (two viable spores and two nonviable); T, tetratype (one nonviable, the remaining viable three showing 2:1 segregation of temperature sensitive to wild-type). (B) Colony formation by cells from a tetratype tetrad, germinated at 36°C for 3 days. WT, wild type (C) Characterization of the myo2-E1 swo1-w1 phenotype. Cells belonging to the swo1-w1 myo2-E1 genotype were rescued by tetrad dissection on medium containing sorbitol and then grown at 24°C in medium containing sorbitol along with strains of wild-type, swo1-w1, and myo2-E1 genotypes. The cells were subsequently shifted to medium lacking sorbitol and stained for nuclei and septa using DAPI and aniline blue, respectively. (D) The restrictive temperature for growth of the double mutant strain swo1-w1 rng3-65 was lowered, with the double mutant being unable to grow at a temperature at which both individual mutant strains still formed colonies. (E) Cells of the rng3-65 strain and the swo1-w1 rng3-65 double mutant strain were grown at 32°C and stained with DAPI and aniline blue to visualize DNA and septa, simultaneously.
cytokinesis and accumulated multiple nuclei before undergoing eventual lysis and death (Fig. 3C). Additionally, swo1-w1 showed a weak interaction with mg3-65, since the double mutants were incapable of colony formation at 32°C, a temperature at which both the parental strains grew and formed colonies (Fig. 3D). Cells belonging to the rng3-65 swo1-w1 strain showed an exacerbated cytokinetic phenotype compared to myo2-S1 cells of the same genotype at 32°C. swo1-w1 cells did not show any strong genetic interaction with myo2-S1, myo2-S2, and 14 other mutants defective in cytokinesis (data not shown): cdc3, cdc4, cdc8, cps1, cdc12, cdc15, cdc7, cdc11, plo1, sid1, sid2, sid4, spg1, and cdc16 (4, 9, 20, 23, 28, 33). The other allele of Hsp90 in S. pombe, i.e., swo1-w1, also showed a similar synthetic interaction with myo2-E1 and rng3-65 (data not shown). Based on the allele-specific genetic interaction with myo2-E1 and the genetic interaction with rng3-65, we concluded that Swo1p was important for myosin II assembly and/or function.

**myo2-E1 and rng3-65 strains show a heightened sensitivity to the Hsp90 inhibitor geldanamycin.** We then used pharmacological approach to independently study the role of Swo1p in myosin II assembly and/or function. The antibiotic geldanamycin specifically binds Hsp90 and disrupts its function (36). We assessed the ability of wild-type, myo2-E1, myo2-S1, myo2-S2, rng3-65, and cdc4-8 cells to form colonies on plates containing a low dose of geldanamycin (2 μg/ml). Interestingly, consistent with the genetic analysis presented above, myo2-E1, swo1-w1, and rng3-65 were sensitive to low doses of geldanamycin and showed only poor growth whereas myo2-S1, myo2-S2, and cdc4-8 were comparable to wild-type cells in their ability to form colonies (Fig. 4A). On treatment with geldanamycin and DAPI staining, wild-type cultures consisted of a population of mono- and binucleate cells (as seen in DMSO-treated cultures), while myo2-E1, swo1-w1, and rng3-65 cultures contained predominantly multinucleate cells with improperly organized division septa (Fig. 4B). DMSO-treated cells belonging to myo2-E1, swo1-w1, and rng3-65 strains did not display a multinucleate phenotype. Based on the genetic and pharmacological analyses, we concluded that Swo1p was important for myosin II structure and function. The genetic interaction between rng3-65 and swo1-w1, as well as the sensitivity of myo2-E1, rng3-65, and swo1-w1 cells to geldanamycin, suggested that Swo1p and Rng3p might collaborate to facilitate myosin II assembly and/or function.

**Swo1p-GFP persists at the actomyosin ring only in the myo2-E1 strain.** Rng3p is related to the myosin II co-chaperone UNC-45p, which, together with Hsp90, prevents aggregation and misfolding of myosin II heads in C. elegans (6). Previous studies with fission yeast have shown that whereas Rng3p-GFP is detected as a weak cytoplasmic signal in wild-type cells, Rng3p-GFP was detected in the improperly formed actomyosin rings in cells harboring the myo2-E1 mutation but not in cells belonging to myo2-S1, myo2-S2, or other cytokinesis mutant strains (39). We therefore characterized the localization of Swo1p that was fused to GFP at its C terminus (as the sole Swo1p copy expressed from its native chromosomal promoter) in wild-type, myo2-E1, myo2-S1, myo2-S2, and 14 other strains that are defective in cytokinesis. As expected, Swo1p-GFP was detected as a strong and diffuse staining throughout the cytoplasm of wild-type cells, indicative of multiple targets that might depend on Swo1p for their folding, assembly, and function. Swo1p-GFP was also observed as a diffuse cytoplasmic signal in heat-arrested myo2-S1 and myo2-S2 cells and cells belonging to the other mutant strains. Interestingly, as with Rng3p-GFP, Swo1p-GFP was detected in the improperly organized actomyosin rings in heat-arrested myo2-E1 cells (Fig. 5A). The rings of Swo1p-GFP also underwent constriction at a semipermissive temperature, consistent with the ability of myo2-E1 cells to undergo actomyosin ring constriction and septum deposition inefficiently under such conditions (Fig. 5B). These studies established that Swo1p is enriched in the actomyosin ring when myosin II function is compromised as a consequence of the myo2-E1 mutant allele.

**Rng3p physically interacts with the Myo2p head domain and with Swo1p.** We next attempted to obtain evidence for a direct physical association of the Myo2p head domain with

![FIG. 4. The mutant strains myo2-E1, mg3-65, and swo1-w1 show a heightened sensitivity to geldanamycin. (A) Tenfold serial dilutions of exponentially growing cells belonging to a wild-type (WT) strain, three allelic strains of the myo2 gene, and the cdc4-8, rng3-65, and swo1-w1 strains were spotted onto YES agar plates in the presence of 2 μg of geldanamycin (GD) per ml or DMSO as control and incubated at 24°C for 3 days. (B) DAPI and aniline blue staining to reveal nuclear number and septa, respectively, in cells of wild-type, swo1-w1, myo2-
E1, and rng3-65 genotypes treated with either DMSO as control or 2 μg of geldanamycin per ml.](http://ec.asm.org/ Downloaded from)
Swo1p and Rng3p. Yeast two-hybrid analysis was used to check if Rng3p directly interacts with Swo1p and with the Myo2p head domain. Rng3p, Swo1p, and the head domain of Myo2p were cloned into yeast Matchmaker vectors, producing fusions to the binding and activation domains of the yeast transcriptional activator GAL4. Expression of the HIS3 reporter activity and levels of β-galactosidase activity (Fig. 6A) indicated that a specific interaction existed between Rng3p and the head domain of Myo2p as well as between Rng3p and Swo1p. Evidence for a direct interaction between the Myo2p head domain and Swo1p was less clear in this assay. While viable His" colonies were observed for this pair of interactors, the level of β-galactosidase activity was below detectable limits. Control experiments involved the independent transformation of DNA-BD/target plasmid alone and AD/target plasmid, as shown in Fig. 6A. These control transformants did not show any β-galactosidase activity and displayed greatly reduced HIS3 activity, ruling out any autonomous reporter gene activation.

To independently confirm the physical association of Myo2p with Rng3p and Swo1p in vivo, we carried out coimmunoprecipitation experiments. Localization of Rng3p and Swo1p to the actomyosin ring in myo2-E1 strains predicted the possibility of a stronger interaction of these proteins in the mutant background. Hence, we looked for evidence of a physical association of Myo2p, Rng3p, and Swo1p in a myo2-E1 strain background. Strains of the genotypes swo1-HA6His myo2-E1 and rng3-GFP myo2-E1 were generated and used for coimmunoprecipitation experiments (Fig. 6B). Both Rng3p-GFP and Swo1-HA were recovered in immune complexes generated using anti-Myo2p antibodies (Fig. 6B, lanes 4 and 9, respectively) but not with control serum (lanes 5 and 11). Furthermore, mutant Myo2p was recovered when immune complexes were generated with antibodies against HA (HA-Swo1p) and GFP (Rng3p-GFP) (lanes 3 and 10, respectively). Cross-reactivity of anti-GFP and anti-HA antibodies to Myo2-E1p was ruled out (lanes 6 and 12). These data indicate that Swo1p and Hsp90p directly interacts with Myo2-E1p in vitro and suggest an association with Myo2p in vivo.

Myo2-E1p levels are reduced in the absence of Swo1p and Rng3p. Given that our data indicate a role for Rng3p and Swo1p in myosin function, we examined the stability of Myo2-E1p in rng3-65 and swo1-w1 mutant cells. It has previously been shown that the levels of myosin in myo2-E1 remain comparable to those in wild-type strains after 5 h of maintaining cultures at the restrictive temperature of 36°C (reference 38 and data not shown). Immunoblotting experiments using lysates from myo2-E1 rng3-65 and myo2-E1 swo1-w1 strains and anti-Myo2p antibodies indicated that the levels of Myo2-E1p in these strains were less than half of that seen in myo2-E1 cells (Fig. 7A and B). We infer, from these data, the involvement of both Rng3p and Swo1p in maintaining the native conformation and stability of Myo2p.

DISCUSSION

The motor protein myosin is of central importance in eukaryotic motility, since it catalyzes diverse processes such as muscle contraction, transport of organelles, cytokinesis, and organization of the actin cytoskeleton. Most members of the myosin superfamily have a heavy chain consisting of a conserved ~ 80-kDa catalytic domain at the N terminus, followed by an α-helical light-chain binding region and a C-terminal rod-like tail domain. Variations within the tail domain and extensions in the N-terminal head region are the basis for sorting individual myosins into various classes. The head domain at the N terminus of myosin contains the sites for nucleotide hydrolysis and actin binding. Binding and hydrolysis of ATP at this domain leads to conformational changes that alter the affinity of myosin for actin, leading to the association-dissociation cycle that is central to force generation by this motor protein. While myosin tail fragments and myosin light chains appear to spontaneously adopt their functional conformation in nonnative systems, the proper folding of head domains of recombinant myosins has not been straightforward. Expression of an embryonic striated muscle myosin II motor domain, with GFP fused at its C terminus, in...
a functionally active form, was possible only in skeletal muscle myocytes and not in kidney epithelial cell lines (10). This indicated a folding dependency of the motor domain on muscle-specific factors.

Myo2p, one of two type II myosins in \textit{S. pombe}, is an essential component of the actomyosin ring that constricts, concomitant with septum deposition, to effect cytokinesis (18, 22). An increasing amount of evidence indicates that Myo2p in \textit{S. pombe} utilizes accessory proteins to facilitate its function. The first such protein, Rng3p, was isolated in the course of a large-scale screen designed to identify genes important for cytokinesis (4). Germinated spores carrying a null mutation for Rng3p were multinucleate with disorganized F-actin patches and defective septa, consistent with a defect in actomyosin ring assembly. A study of Rng3p localization in \textit{S. pombe} indicated that Rng3p-GFP was detected only in improperly assembled actomyosin rings at the restrictive temperature in \textit{myo2-E1} mutants and not in wild-type, \textit{myo2-S1}, \textit{myo2-S2}, or 18 other cytokinesis mutants (39). The \textit{myo2-E1} mutation is a G345R point mutation in the head region of Myo2p between the F-actin and ATP-binding domains. Similarly, two \textit{rng3} mutant alleles, \textit{rng3-65} and \textit{rng3-A3}, displayed synthetic lethality with \textit{myo2-E1} but not with 19 other mutants including the other 2 myosin mutant alleles, mutants defective in actomyosin ring assembly and placement, and mutants defective in regulation of septum synthesis (39). Rng3p was shown to be necessary for localization and maintenance of the type II myosin in \textit{S. pombe}, Myo2p, in a spot structure that behaves as a progenitor for actomyosin ring formation (38).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Physical interactions between Myo2p and Rng3p with Swo1p. (A) Cell suspensions of the \textit{S. cerevisiae} strain Y190 carrying the indicated BD and AD plasmids were selected on synthetic complete medium lacking leucine and tryptophan and rescreened for HIS\textsuperscript{+} expression to select for strains containing pairs of interacting proteins. Colonies grown on histidine-minus medium with 3-aminotriazole (35 mM) were assayed for \(\beta\)-galactosidase activity by a colony lift filter assay. (B) Cell extracts were prepared from \textit{myo2-E1}, \textit{myo2-E1 swo1-HAH}, and \textit{myo2-E1 rng3-GFP} strains and immunoprecipitated (IP) with anti-Myo2p antibodies, anti-HA antibodies, anti-GFP antibodies, or nonspecific control antibodies. Lanes: 1, \textit{myo2-E1} lysate; 2, \textit{myo2-E1 swo1-HAH} lysate; 3, \textit{myo2-E1 swo1-HAH} IP with anti-HA antibodies; 4, \textit{myo2-E1 swo1-HAH} IP with anti-Myo2p antibodies; 5, \textit{myo2-E1 swo1-HAH} IP with nonspecific immunoglobulin G; 6, \textit{myo2-E1} IP with anti-HA antibodies; 7, \textit{myo2-E1} lysate; 8, \textit{myo2-E1 rng3-GFP} lysate; 9, \textit{myo2-E1 rng3-GFP} IP with anti-Myo2p antibodies; 10, \textit{myo2-E1 rng3-GFP} IP with anti-GFP antibodies; 11, \textit{myo2-E1 rng3-GFP} IP with nonspecific IgG; 12, \textit{myo2-E1} IP with anti-GFP antibodies.}
\end{figure}
Swo1p and present the first in vivo data indicating that Swo1p mutant allele of the fission yeast Hsp90-encoding gene, Myo2p structure and function in the absence of hydroxyurea (38, 39). actomyosin ring on release from the restrictive temperature in treatment with hydroxyurea, and subsequent failure to form the Myo2p progenitor spot at restrictive temperatures on treatment with hydroxyurea (34). Geldanamycin treatment of C2C12 myocytes caused the formation of aggregates which contained myosin, Hsp90, and Hsc70, as detected by immunostaining with specific antibodies.

Our results substantiate in vitro studies performed using unc-45, the mg3 homolog in C. elegans. The UNC-45 protein is found throughout the thick filaments of body wall muscle sarcomeres (2) and has an N-terminal domain composed of three tetratricopeptide repeat (TPR) motifs and a C-terminal UCS domain (7, 35). In vitro binding assays revealed that UNC-45 formed stoichiometric complexes with scallop myosin head subfragment 1 (S1) and Hsp90, suggesting the formation of a ternary complex in which myosin binds the C-terminal region and Hsp90 binds the N-terminal region of UNC-45 (7). Evidence, using the S. pombe system, for a physical interaction between Rng3p and the head domain of Myo2p as well as between Rng3p and Swo1p was obtained using the yeast two-hybrid system and coimmunoprecipitation experiments with myo2-E1 mutant strains. Recent studies by Lord and Pollard (21) have also shown that S. pombe Rng3p physically interacts with Myo2p and might stimulate its activity. Our results also suggest that Rng3p and Swo1p is important for the stability of Myo2p. The mode of chaperone-assisted folding of the Myo2p head in S. pombe is still open to speculation. Hsp90 in mammalian cells interacts with a host of other cochaperones that facilitate its interaction with nucleotides and client proteins (summarized on the webpage http://www.picard.ch/DP/downloads/Hsp90interactors.pdf). The cochaperones are divided into two classes based on the presence or absence of TPR domains that bind at the C-terminal MEEVD motif in Hsp90. Some of these cochaperones additionally interact with the client proteins and with each other. Rng3p in S. pombe lacks a TPR domain. The binding of Swo1p to Rng3p may therefore be either at an alternate region of Rng3p or via a hitherto unidentified protein. Future work will involve the identification and characterization of TPR domain-containing proteins and proteins displaying functional cooperation with Swo1p in S. pombe, with a view to characterizing the entire subset of proteins involved in folding the myosin head domain into its proper conformation.

ACKNOWLEDGMENTS

We are grateful to Juan Jimenez for providing the swo1-w1 strain, to Paul Russell for providing the swo1-26 and swo1-HA strains, to Keith Gull for providing the TAT-1 antibodies, and to Er Poh Nee (senior imaging facility engineer at Temasek Life Sciences Laboratory) for guidance with confocal microscopy. We thank all members of the yeast and fungal biology laboratories at the Temasek Life Sciences Laboratories for encouragement and advice.

This work was supported by research funds from the Temasek Life Sciences Laboratory.

FIG. 7. Swo1p and Rng3p are required for the stability of myo2-E1p. (A) Lysates of the indicated strains were arrested at the restrictive temperature of 36°C for 5 h, resolved by SDS-PAGE (12% polyacrylamide), immunoblotted, and probed with antibodies against Myo2p and Arp3p. (B) The amount of myosin in the three strains as quantified by densitometry, after background subtraction and normalization against Arp3p band intensity, was estimated and plotted.

myosin molecules having potentially misfolded head domains. An interaction between Swo1p and Rng3p with the Myo2p head domain may be too transient and/or diffuse to be detected in wild-type cells by fluorescence microscopy. Our evidence for chaperone-mediated folding of the myosin head domain is consistent with the results of immunofluorescence studies of differentiating C2C12 myocytes, which have revealed that GFP-tagged embryonic myosin heavy chain colocalizes with the molecular chaperones Hsc70 and Hsp90 in intermediates but not in the mature myofibrils (34). Geldanamycin treatment of C2C12 myocytes caused the formation of aggregates which contained myosin, Hsp90, and Hsc70, as detected by immunostaining with specific antibodies.

We now report the involvement of a second protein in Myo2p structure and function in S. pombe, the Hsp90 homolog Swo1p, and present the first in vivo data indicating that Swo1p and Rng3p cooperate in proper Myo2p function in the fission yeast S. pombe. Our characterization of the phenotype of a mutant allele of the fission yeast Hsp90-encoding gene, swo1-w1, reveals that the swo1-w1 mutant is defective in actomyosin ring assembly. The swo1-w1 mutant shows phenotypic similarities to myo2Δ and rng3Δ mutants. This evidence, combined with allele-specific genetic interactions between myo2-E1 and swo1-w1 as well as between rng3-65 and swo1-w1 mutant pairs, suggests that Swo1p probably participates in myosin II assembly and/or function in vivo. Moreover, partial reduction of Swo1p function by pharmacological treatment with the Hsp90 inhibitor geldanamycin resulted in severe cytokinesis defects in myo2-E1, rng3-65 cells, and swo1-w1 cells but did not affect myo2-S1, myo2-S2, cdc4-8, and wild-type cells at the concentrations used. As was observed with Rng3p, Swo1p-GFP has been detected in the improperly formed actomyosin rings in a myo2-E1 strain but not in wild-type cells. This observed colocalization may be attributed to their persistent association with
REFERENCES


