Class I myosins function in cell motility, intracellular vesicle trafficking and endocytosis. Recently, it was shown that class I myosins are phosphorylated by a member of the p21-activated kinase (PAK) family. PAK phosphorylates a conserved serine or threonine residue in the myosin heavy chain. Phosphorylation at this site is required for maximal activation of the actin-activated Mg\(^{2+}\)-ATPase activity in vitro. This serine or threonine residue is conserved in all known class I myosins of microbial origin and in the human and mouse class VI myosins. We have investigated the in vivo significance of this phosphorylation by mutating serine 371 of the class I myosin heavy chain gene myoA of Aspergillus nidulans. Mutation to glutamic acid, which mimics phosphorylation and therefore activation of the myosin, results in an accumulation of membranes in growing hyphae. This accumulation of membranes results from an activation of endocytosis. In contrast, mutation of serine 371 to alanine had no discernible effect on endocytosis. These studies are the first to demonstrate the in vivo significance of a regulatory phosphorylation on a class I myosin. Furthermore, our results suggest that MYOA has two functions, one dependent and one independent of phosphorylation.

Class I myosins have been implicated in a variety of cellular processes, including cell locomotion, contractile vacuole function, receptor-mediated endocytosis, protein secretion, and intracellular vesicle transport (1). We have been studying the function of the class I myosin gene, myoA, of the filamentous fungus Aspergillus nidulans (2). We constructed a conditional null mutant myoA strain using the promoter of the inducible alcohol dehydrogenase gene alcA (2). Using this strain, we showed that myoA is an essential gene in A. nidulans and that it was required for the establishment of polarized hyphal growth. We also showed that strains lacking myoA were deficient for the secretion of acid phosphatase into the medium. In contrast, work on class I myosin function in the budding yeast Saccharomyces cerevisiae has shown that they function in endocytosis and possibly protein secretion (3, 4). In S. cerevisiae there are two genes, MYO3 and MYO5, that code for class I myosin polypeptides. Deletion of either of these genes is not lethal, suggesting that they have overlapping or redundant functions. Deletion of both genes produces strains that either grow poorly (4) or are inviable (3). A strain that has a temperature-sensitive myoA mutation is defective for endocytosis but not for protein secretion (3).

Studies of class I myosin function and their actin-activated ATPase activity in Dictyostelium discoideum and Acanthamoeba castellani have shown that these class I myosins are regulated by phosphorylation at a specific and highly conserved serine or threonine residue on the heavy chain (5, 6). This phosphorylation site is also found in other unconventional myosins such as the class VI myosins from humans and mice (Fig. 1). Recent studies have shown that the kinase responsible for phosphorylation of the amoeboid class I myosins is a member of the PAK/STE20 family of serine-threonine protein kinases (5, 6). Binding of the GTP-bound form of the small GTPases, Rac or Cdc42 (7, 8), stimulates the activity of PAKs. Activated forms of these small GTPases when transfected into tissue culture cells have dramatic effects on the organization of the actin cytoskeleton (7, 8).

The demonstration that a PAK regulates the activity of class I myosin provides a link to how members of the small GTP-binding proteins, like Rac, mediate some of their effects on the actin cytoskeleton and membrane ruffling of vertebrate cells in culture (7–9). Class I or other unconventional myosins, like the class VI myosins, may in turn be the functional connection between the regulatory activity of PAKs and the motor activity necessary for changes in the actin cytoskeleton and membrane ruffling. The morphological changes previously attributed to PAKs may in fact be due to phosphorylation of myosins by PAK-like kinases.

To investigate the in vivo significance of phosphorylation of class I myosin heavy chain in myosin function, we have mutated the class I myosin gene myoA of A. nidulans and examined the consequences of these changes on cell growth, cell morphology, protein secretion, and endocytosis. We made these mutants because in vitro the myosin I kinase from A. castellani phosphorlates a synthetic peptide with the sequence of this region of MYOA. We generated mutant forms of MYOA containing either alanine or glutamic acid substitutions at the conserved, PAK-phosphorylated serine 371 of MYOA (2). These mutations were introduced back into the chromosomal myoA locus by homologous recombination. Strains were identified that express only the mutant forms of the class I myosin (S371E or S371A), and the phenotypes of these strains were characterized. Our results indicate that mutation of serine 371 to glutamic acid leads to constitutive activation of endocytosis.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis—*S371A and S371E myoA mutations were made using a polymerase chain reaction (PCR)-based mutagenesis method (10). The names and sequences of the primers used in these studies are given in Table 1.

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† H. Brzeska and E. Korn, personal communication.
Myosin I and Endocytosis in Aspergillus nidulans

Table I

| Primer name | Sequence |
|-------------|----------|
| myoA3       | CGTCCGGTCTAATA |
| myoA5       | TGTTAATAGTGA |
| myoA7       | TTTCATCGATTA |
| myoA24      | ATCATGGTGAACCTG |
| Ser-Ala     | GCTGTTGCGCGCTTTAG |
| Ser-Ala*    | CTATCAAGGCCGCCCCAG |
| Ser-Glu     | GCCCTGGCCGGGGTTTATAG |
| Ser-Glu*    | CTATCAAGGCCGCCCCAG |

The conserved serine or threonine residue that can be phosphorylated is bolded and is underlined. The single letter amino acid code has been used. Conservative amino acid substitutions are indicated by a +, and identities are indicated by the letter. All similarities are derived from comparisons to the A. nidulans MYOA. Abbreviations for the sequences used to make the alignment are: MYOA, A. nidulans; MYO3, S. cerevisiae; MYO5, S. cerevisiae; DDIC, D. discoideum IC; ACIB, A. castellanii IB; MusVI, mouse myosin VI; and HumVI, human myosin VI.

Table I. The primers used to make the mutations in myoA were Ser-Ala, Ser-Ala*, Ser-Glu, and Ser-Glu*. Ser-Ala and Ser-Glu were phosphorylated with T4 polymerase kinase prior to being used in the PCR. The flanking primers used were myoA3, and myoA7. The conditions for the PCR reaction were 95 °C for 5 min to denature the plasmid. This was followed by 30 cycles of 95, 55, and 72 °C for 1, 1, and 2 min, respectively. The reactions consisted of 10 ng of plasmid DNA template in 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, 0.2 mM deoxynucleotide triphosphates, 0.1 mg/ml gelatin, and 100 ng of the appropriate primer pair in a final volume of 0.05 ml. The final PCR product was digested with NheI and MluI and cloned into plasmid myo4. The presence of the specific mutations and the absence of other changes were confirmed by DNA sequencing. A 15-1911 fragment containing the mutation was used to replace the equivalent wild type sequence in the plasmid myo3 to generate myo3-Ala and myo3-Glu. These plasmids are based on the vector pRG3 and used for transformation into A. nidulans (13). The PCR conditions to determine which myoA allele was being expressed used the same conditions described above but used 100 ng of genomic DNA as template in the first PCR reaction. The primers used in the first PCR reaction were myoA5 and myoA7. The product of this reaction was 1274 bp (base pairs) in length. The primer myoA5 is not contained in the sequences of the integrative vector and would not produce a PCR product with the expected restriction polymorphism unless the crossover during homologous recombination was upstream of the site of the mutation. Thus, non-homologous integration events and those in which the crossover occurred downstream of the site of the mutation would yield the wild type sequence lacking the restriction polymorphism expected for the mutant allele. The product from the first PCR reaction was purified using a Qiaquick PCR purification kit (Qiagen, Inc.). A portion of the purified PCR product was used to prime a second reaction with the primers myoA24 and myoA7 to produce a product of 190 bp. The time of incubation at each temperature were reduced by half for this second PCR reaction.

Microscopy—Differential interference contrast micrographs were collected from hyphal tips grown in 0.5% yeast extract, 20 m M glucose for 20 h at room temperature. The S371A and S371E mutants and a control strain were isolated from hyphal tips grown in 0.5% yeast extract, 1% dextrose, and 20 m M glucose for 20 h at room temperature. The S371A and S371E mutations were generated on strains expressing the mutant allele to confirm that a single integration event had occurred at the homologous myoA locus (data not shown). The phenotype of the S371A and S371E mutations was confirmed for at least three independent isolates.

Detection of the S371A and S371E Mutations in the Genome—Primary transformants were purified three times to single spores on selective medium. A PCR-based assay was designed to determine whether the wild type or mutant allele of myoA was being expressed (Fig. 2A). Our assay uses two rounds of PCR to detect the allele being expressed from the promoter of myoA. Both the S371A and S371E mutations generate a restriction polymorphism that was readily identified following PCR amplification from the expressed gene. The S371A mutation introduces a HaeII site, which results in two fragments of 105 and 85 bp after digestion of the PCR product. The S371E mutation introduces a BstI site, which produces three fragments of 105, 59, and 26 bp while the wild type produces two fragments of 131 and 59 bp (Fig. 2B). The nested PCR procedure made detection of the restriction polymorphism reproducible. Genomic Southern analysis was performed on strains expressing the mutant allele to confirm that a single integration event had occurred at the homologous myoA locus (data not shown). The phenotype of the S371A and S371E mutations was confirmed for at least three independent isolates.

The S371E Mutant Accumulates Intracellular Membranes—The S371A and S371E mutants and a control strain were examined by light microscopy. We found that the S371E mutation had a dramatic effect on the appearance of the cytoplasm by differential interference contrast (DIC) microscopy. Hyphal tips of the S371E mutant strain displayed a grainy appearance, suggestive of a major change in the composition of the cytoplasm, whereas the hyphal tips of the S371A and control strains appeared similar to one another (Fig. 3). Many things could account for the differences in the appearance of the cytoplasm between the control strains and the S371E mutant strain. Among these would be changes in organization of cytoskeletal elements, the accumulation of additional membranous organelles, or a change in the amount of solute in the cytoplasm.

To determine the reason for the changes in the cytoplasm, we examined hyphae by electron microscopy. Thin section electron micrographs of control and S371E mutant hyphae confirmed our light microscopic observations, suggesting a change in the organization of the cytoplasm (Fig. 4). Vesicles were visible in the tips of the control and S371A strains, but they were present in greater numbers along with tracks of membranes, presumably formed by invagination, in the S371E mutant. In addition to the membrane tracks in the S371E mutant, other intracellular membranes take on a rougher appearance (Fig. 4). To show that this difference is not restricted to those images we
with the restriction endonuclease.

or Bsl were then digested (lanes are DNA products from the PCR reaction. The DNA fragments were not treated with the restriction endonuclease.

In yeast, class I myosin has been shown to be present, we quantified the difference in the numbers of vesicles and membrane tracks in the control and mutant strains. The number of vesicles and membrane tracks was determined for a fixed area in eight hyphal tips from the control and the two mutant strains. The S371A mutant strain had 70% of the vesicles and 75% of the membrane tracks observed in the control strain. In contrast, the S371E mutant strain had 2.6 times the number of vesicles and membrane tracks in the control and mutant strains. The S371A mutant strain had 70% of the number of vesicles and membrane tracks was determined for a sufficient growth period. The S371A and S371E mutations had no discernible effects on the growth of the fungus on normal solid media. In contrast to the apparently normal growth observed for these strains on standard solid media, we noticed that the mutant strains grew less well in submerged liquid cultures than did the control strains. Both the S371E and S371A mutant strains took longer to germinate, as measured by the timing of germtube emergence. At 5 h of growth, approximately 20% of the control spores and less than 2% of the mutant spores had germinated. Similarly, both mutants showed delays in sending out a second germtube from the conidium and in hyphal branching when compared with control strains. Though the mutants grew more slowly than control strains they were morphologically normal and were able to reach the same hyphal mass in culture given a sufficient growth period.

We also noted that both the S371A and S371E mutant strains appeared to conidiate less well on solid medium containing high concentrations of potassium chloride. We quantified the effect of higher potassium chloride on conidiation in the S371A and S371E strains by determining the number of conidia produced by each of the mutant strains and a control wild type strain. The S371A and S371E mutations had no discernible effects on the growth of the fungus on normal solid media. In contrast to the apparently normal growth observed for these strains on standard solid media, we noticed that the mutant strains grew less well in submerged liquid cultures than did the control strains. Both the S371E and S371A mutant strains took longer to germinate, as measured by the timing of germtube emergence. At 5 h of growth, approximately 20% of the control spores and less than 2% of the mutant spores had germinated. Similarly, both mutants showed delays in sending out a second germtube from the conidium and in hyphal branching when compared with control strains. Though the mutants grew more slowly than control strains they were morphologically normal and were able to reach the same hyphal mass in culture given a sufficient growth period.

The Effects of the S371A and S371E Mutations on Growth, Development, and Protein Secretion—The growth characteristics of strains identified as expressing only the S371A or S371E mutant forms of MYOA were examined under a variety of conditions. The S371A and S371E mutations had no discernible effects on the growth of the fungus on normal solid media. In contrast to the apparently normal growth observed for these strains on standard solid media, we noticed that the mutant strains grew less well in submerged liquid cultures than did the control strains. Both the S371E and S371A mutant strains took longer to germinate, as measured by the timing of germtube emergence. At 5 h of growth, approximately 20% of the control spores and less than 2% of the mutant spores had germinated. Similarly, both mutants showed delays in sending out a second germtube from the conidium and in hyphal branching when compared with control strains. Though the mutants grew more slowly than control strains they were morphologically normal and were able to reach the same hyphal mass in culture given a sufficient growth period.

We also noted that both the S371A and S371E mutant strains appeared to conidiate less well on solid medium containing high concentrations of potassium chloride. We quantified the effect of higher potassium chloride on conidiation in the S371A and S371E strains by determining the number of conidia produced by each of the mutant strains and a control wild type strain on medium with increasing concentrations of potassium chloride (Fig. 6). The S371E mutant displayed the most dramatic response to the presence of increasing concentrations of potassium chloride in the medium. Conidiation in the S371E mutant strain was 70% of that observed for the control on 0.3 M potassium chloride. Conidiation in this mutant strain remained at 50–70% of the control strain at potassium chloride concentrations up to 1.2 M. Similarly, the S371A mutant strain showed reduced levels of conidiation but not until the concentration of potassium chloride reached 0.9 M or higher. The effect of potassium chloride on conidiation was not simply the result of increased osmolarity of the medium because a similar effect on conidiation was not seen when sucrose was substituted for potassium. We conclude from this that efficient conidial development in high medium requires that the activity of MYOA be precisely regulated.

Previously, we showed that protein secretion was reduced in patches that continued to increase in number out to 20 min. In contrast, the number and size of fluorescent patches in the control were reduced at all time points. Thus, the S371E mutant strain showed a dramatic increase in plasma membrane internalization. To demonstrate that this internalization of plasma membranes was F-actin dependent, we treated germlings with cytochalasin D, a compound that depolymerizes F-actin. Cytochalasin D treatment of control and S371E mutant germlings blocked FM 4–64 internalization (Fig. 5). Together these experiments demonstrate that FM 4–64 uptake is a MYOA-dependent process that also requires F-actin.

The S371E Mutant Endocytosis Phenotype Is Dominant—We predicted that the S371E mutation resulted in a constitutively active myosin, and therefore the phenotype would be dominant. We tested this hypothesis by constructing a diploid between an S371E mutant and a control strain, FGSC 122 (Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS). Diploids heterozygous for the wild type and S371E MYOA proteins displayed the same granary appearance of the cytoplasm characteristic of the S371E myoA mutant strains when examined by DIC microscopy (data not shown). Thus, constitutive activation of MYOA function by mutation of serine 371 to glutamic acid leads to a dominant activation of endocytosis.

Intracellular Membranes Accumulate in the S371E Mutant by Endocytosis—In yeast, class I myosin has been shown to be important for endocytosis (3). Therefore, we hypothesized that the S371E mutant internalizes more plasma membrane by endocytosis. To test this, we examined endocytosis in germlings of the control and the S371A and S371E mutants by measuring the uptake of the fluorescent molecule FM 4–64. FM 4–64 is a lipophilic styryl dye that has been used to follow bulk membrane internalization and transport in yeast (11). Germlings were incubated in FM 4–64 at 0 °C to label the plasma membrane and then warmed to room temperature. Internalization of the dye was assessed at various times by fluorescence microscopy. We observed that FM 4–64 was rapidly internalized in S371E germlings, whereas in the control its internalization was much slower (Fig. 5). After 10 min, the internalized membrane in the S371E mutant appeared as large fluorescent patches that continued to increase in number out to 20 min. In contrast, the number and size of fluorescent patches in the control were reduced at all time points. Thus, the S371E mutant strain showed a dramatic increase in plasma membrane internalization. To demonstrate that this internalization of plasma membranes was F-actin dependent, we treated germlings with cytochalasin D, a compound that depolymerizes F-actin. Cytochalasin D treatment of control and S371E mutant germlings blocked FM 4–64 internalization (Fig. 5). Together these experiments demonstrate that FM 4–64 uptake is a MYOA-dependent process that also requires F-actin.

The Effects of the S371A and S371E Mutations on Growth, Development, and Protein Secretion—The growth characteristics of strains identified as expressing only the S371A or S371E mutant forms of MYOA were examined under a variety of conditions. The S371A and S371E mutations had no discernible effects on the growth of the fungus on normal solid media. In contrast to the apparently normal growth observed for these strains on standard solid media, we noticed that the mutant strains grew less well in submerged liquid cultures than did the control strains. Both the S371E and S371A mutant strains took longer to germinate, as measured by the timing of germtube emergence. At 5 h of growth, approximately 20% of the control spores and less than 2% of the mutant spores had germinated. Similarly, both mutants showed delays in sending out a second germtube from the conidium and in hyphal branching when compared with control strains. Though the mutants grew more slowly than control strains they were morphologically normal and were able to reach the same hyphal mass in culture given a sufficient growth period.

We also noted that both the S371A and S371E mutant strains appeared to conidiate less well on solid medium containing high concentrations of potassium chloride. We quantified the effect of higher potassium chloride on conidiation in the S371A and S371E strains by determining the number of conidia produced by each of the mutant strains and a control wild type strain on medium with increasing concentrations of potassium chloride (Fig. 6). The S371E mutant displayed the most dramatic response to the presence of increasing concentrations of potassium chloride in the medium. Conidiation in the S371E mutant strain was 70% of that observed for the control on 0.3 M potassium chloride. Conidiation in this mutant strain remained at 50–70% of the control strain at potassium chloride concentrations up to 1.2 M. Similarly, the S371A mutant strain showed reduced levels of conidiation but not until the concentration of potassium chloride reached 0.9 M or higher. The effect of potassium chloride on conidiation was not simply the result of increased osmolarity of the medium because a similar effect on conidiation was not seen when sucrose was substituted for potassium. We conclude from this that efficient conidial development in high medium requires that the activity of MYOA be precisely regulated.

Previously, we showed that protein secretion was reduced in
the absence of MYOA function (2). For this reason we looked at the secretion of invertase and acid and alkaline phosphatases. The secretion of all three enzymes was not significantly different from that of control strains when normalized for cell mass. Thus, the S371A and S371E mutations do not have a measurable effect on protein secretion (data not shown).

DISCUSSION

The experiments are the first to investigate fungal class I myosin function in vivo. We have shown that the phosphorylation site for a PAK, serine 371 of the MYOA protein, plays a central role in regulating class I myosin activity in vivo. Mutation of serine 371 to glutamic acid leads to constitutive activation of endocytosis in A. nidulans. Using both light and electron microscopy, we showed that plasma membrane is internalized more rapidly and to a greater degree in the S371E mutant strains. We also showed that membrane internalization in the S371E and control strains requires the presence of filamentous actin, as would be predicted if MYOA was functioning as the motor for membrane internalization.

We previously reported that, in A. nidulans, the class I myosin, MYOA, functioned in polarized hyphal growth and protein secretion (2). The studies we report here suggest a role for class I myosin in endocytosis. We attribute the differences observed in our previous studies and those in budding yeast (3) to the kinds of mutations used in the various studies. Our earlier studies which suggested that MYOA functions in polarized cell growth and secretion used a conditional null mutant

FIG. 3. Differential interference contrast micrographs of hyphal tips of a control strain, an S371A mutant strain, and an S371E mutant strain. We always observe the difference in the appearance of the cytoplasm between the control and the S371E mutant strains. The bar in the lower left panel is 5 µm long.

FIG. 4. Thin section electron micrographs (11) of hyphal tips of a control strain (A), an S371A mutant strain (B), and an S371E mutant strain (C and D). The bar in panel D is 1 µm long.

FIG. 5. Fluorescence micrographs of a control strain and an S371E strain stained with the lipophilic dye FM 4–64 on ice and imaged at 0, 10, and 20 min after transfer to room temperature. The two panels at the far right are the control and S371E treated in the same manner but in the presence of cytochalasin D after 15 min. We always see this difference in FM 4–64 uptake between the control and the S371E mutant strains. The bar in the lower left panel is 5 µm long.

FIG. 6. Growth on medium with increasing concentrations of potassium chloride reduces the number of conidia produced by the S371A and S371E mutant strains relative to a control strain.
strains, whereas our current studies used a point mutation that would not be expected to grossly interfere with MYOA function. Like our present studies, the studies in yeast were conducted with a point mutation that confers heat-sensitive growth (3). We would suggest that the divergent results obtained are the consequence of the kind of mutation used to conduct the studies. Our previous studies were conducted with a conditional null mutation in myoa, whereas our current studies use point mutants. Thus, the complete absence of a MYOA polypeptide has a more dramatic phenotype than that observed for the point mutants. We propose that, in the absence of MYOA polypeptide, cell polarity cannot be established and that this is necessary for efficient growth and protein secretion. In contrast, if a MYOA polypeptide is made, even if it is somewhat defective as in this study, the establishment of cell polarity is not impaired and protein secretion is normal. So why is the S371A mutant viable if one requires activation of MYOA by phosphorylation by a PAK? One possibility is that the S371A mutation is not inactivating and that basal activity is sufficient to support growth of the fungus. Alternatively, MYOA motor function is not the essential activity and there are other transport systems that overlap those of MYOA in endocytosis. Thus MYOA has two functions, it is a motor protein functioning in endocytosis, a nonessential activity of the myosin, and it is an actin-binding protein that is required for polarized hyphal growth and protein secretion. In yeast, many mutations that alter the actin cytoskeleton have effects on bud site selection and the directed transport of chitin to the growing cell wall, invertase secretion, endocytosis, and mitochondrial distribution (4, 15–25). These processes are similar to polarized hyphal growth and protein transport for secretion.

The current studies are the first to establish that the PAK phosphorylation site can play a significant role in regulating class I myosin function in vivo. It further demonstrates that activation of class I myosin leads to increased endocytic activity. Since small GTP-binding proteins of the Rho family activate PAKs and activated forms of these small GTP-binding proteins have dramatic effects on the organization of the actin cytoskeleton, it is interesting to speculate that at least some of the changes are mediated through activation of unconventional myosins. One inherent problem with this hypothesis is that class I myosins of vertebrates lack the conserved serine or threonine residue at the site of phosphorylation. Instead, they have a charged amino acid that would closely mimic the S371E mutant we have used in this study (26). This makes it unlikely that vertebrate class I myosins are regulated by a p21-activated kinase. A more likely target for regulation by a PAK is the class VI unconventional myosin family. The class VI myosins have the conserved serine or threonine at the PAK phosphorylation site and thus could have their activity regulated by phosphorylation (Fig. 1). We know of no current studies that demonstrate that a class VI myosin family member is the substrate for a PAK. It would be interesting to see what consequences transfection of a class VI myosin that has its conserved serine or threonine residue mutated to a charged amino acid is on the actin cytoskeleton of a vertebrate cell in culture.

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