Two type V myosins with non-overlapping functions in the fission yeast *Schizosaccharomyces pombe*: Myo52 is concerned with growth polarity and cytokinesis, Myo51 is a component of the cytokinetic actin ring

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SUMMARY

The fission yeast genome project has identified five myosin genes: one type I myosin, *myo1*$^+$, two type II myosins, *myo2*$^+$ and *myp2*$^+$, and two type V myosins, *myo51*$^+$ and *myo52*$^+$. Cells deleted for *myo51*$^+$ show normal morphology and growth rates whereas deletion of *myo52*$^+$ results in a partial loss of cell polarity, slow growth and cytokinetic defects. Combining both deletions in a single strain is phenotypically non-additive, *myo52Δ* being epistatic to *myo51Δ*. Overproduction of Myo51 gives rise to elongated cells which fail to form functional septa whereas overproduction of Myo52 results in branched cells with aberrant septa that fail to cleave. Myo52 localises to the poles of growing cells but during cell division it relocalises to the cell equator as a bar that is bisected by the cytokinetic septum. Myo51 shows no obvious localisation during interphase but at cytokinesis it is associated with the contractile cytokinetic actin ring (CAR). Both myosins are dependent upon an intact actin cytoskeleton for localisation. Myo52 partially colocalises with the α-glucan synthase Mok1 at the cell tips and to a lesser extent at the septum. Mok1 is delocalised and upregulated in *myo52Δ* and *myo52Δ* cell walls are resistant to digestion by the cell wall degrading enzyme zymolyase. Thus *myo52*$^+$ appears to be involved in the local delivery or positioning of vesicles containing cell wall precursors at the cell tips and has a role in the maturation or cleavage of the septum. Myo51 has a non-essential role in cytokinesis as a component of the cytokinetic actin ring.

Key words: Myosin V, Fission yeast, Growth polarity, Cytokinesis, Cell wall

INTRODUCTION

Eukaryotic cells possess two distinct mechanisms for generating force; a long-range mechanism based on microtubules and the motor proteins dynein and kinesin and a short-range actin-based system powered by the motor protein myosin. These two systems act cooperatively (Goode et al., 2000). Certain classes of vesicles are dependent for both actin and microtubule tracks for their dispersal (Rogers and Gelfand, 1998; Rodionov et al., 1998) and myosin and kinesin motors have been shown to interact physically (Huang et al., 1999; Beningo et al., 2000). At least 15 classes of myosin are now recognised and cells express different combinations of myosin types appropriate to their functions (Baker and Titus, 1998; Mermall et al., 1998). Non-motile, unicellular organisms such as yeasts probably express a minimum subset of myosins required to maintain basic cellular functions. Budding yeast possesses a single gene encoding a conventional type II myosin (encoded by the gene *MYO1*; Watts et al., 1987; Sweeney et al., 1990) and two genes encoding the unconventional type I (*MYO3* and *MYO5*; Goodson and Spudich, 1995; Goodson et al., 1996) and type V myosins (*MYO2* and *MYO4*; Johnston et al., 1991; Haarer et al., 1994). Myo2p and Myo4p, have distinct cellular functions. Myo2p is a multifunctional motor that is implicated in the delivery of secretory vesicles to the bud (Johnston et al., 1991; Govindan et al., 1995; Pruyne et al., 1998; Schott et al., 1999), in the selection of the bud site via the localisation of chitin synthase 3 (Santos and Snyder, 1997), in the movement of a portion of the vacuole to the daughter cell (Hill et al., 1996; Catlett and Weisman, 1998; Reck-Peterson et al., 1999; Catlett et al., 2000) and, through its interaction with the microtubule-binding protein Kar9p, in spindle orientation (Yin et al., 2000). Myo4p on the other hand is required for mating type switching. Myo4p is believed to deliver the mRNA for *ASH1* (for asymmetric synthesis of HO) to the bud tip. Ash1p inhibits expression of the *HO* endonuclease in daughter cells thus ensuring that mating type switching is confined to the mother cell (Bobola et al., 1996; Jansen et al., 1996; Long et al., 1997; Takizawa et al., 1997; Bertrand et al., 1998). Nothing is known about the regulation

Table 1. Strains used in this study

Genotype	Source
ade6-210 leu1-32 ura4-D18 h⁻/ade6-216 leu1-32 ura4-D18 h⁺	P. Fantes
ade6-210 leu1-32 ura4-D18 h⁻	P. Fantes
ade6-216 leu1-32 ura4-D18 h⁺	P. Fantes
myo51::ura4⁺ ade6-210 leu1-32 ura4-D18 h⁻/ade6-216 leu1-32 ura4-D18 h⁺	This study
myo51::ura4⁺ ade6-216 leu1-32 ura4-D18 h⁺	This study
myo51-GFP^{S65T} ade6-210 leu1-32 ura4-D18 h⁻ (myo51-gc)	This study
myo52::ura4⁺ ade6-210 leu1-32 ura4-D18 h⁻/ade6-216 leu1-32 ura4-D18 h⁺	This study
myo52::ura4⁺ ade6-216 leu1-32 ura4-D18 h⁻	This study
myo52::ura4⁺ ade6-216 leu1-32 ura4-D18 h⁺	This study
myo52-GFP^{S65T} ade6-210 leu1-32 ura4-D18 h⁻ (myo52-gc)	This study
myo51::ura4⁺ myo52::ura4⁺ ade6-216 leu1-32 ura4-D18 h⁻	This study
myo51::ura4⁺ cps8⁻ ura4-D18 h⁻	This study
myo52::ura4⁺ cps8⁻ ura4-D18 h⁻	This study
cps8⁻ ura4-D18 h⁻	J. Ishiguro
myp2::ura4⁺ ade6-210 leu1-32 ura4-D18 h⁺	May et al., 1998
mok1-664 leu1-32 h⁻	T. Toda
pck2::LEU2 leu1-32 h⁻	T. Toda
cdc25-22 leu1-32 h⁻	P. Fantes

of Myo4p but Myo2p appears to be associated with two light chains, calmodulin (Brockerhoff et al., 1994; Sekiya-Kawasaki et al., 1998) and a novel myosin light chain, Mlc1p (Stevens and Davis, 1998) which may additionally regulate the IQGAP protein Iqg1p (Epp and Chant, 1997; Lippincott and Li, 1998). Myo2p also interacts with a kinesin-like protein Smy1p (Lillie and Brown, 1992; Lillie and Brown, 1994; Lillie and Brown, 1998; Beningo et al., 2000).

We have begun to characterise the myosin family in the fission yeast, *Schizosaccharomyces pombe*. As in budding yeast, the fission yeast genome also contains representatives of the myosin classes I, II and V. Unlike budding yeast, *S. pombe* possesses a single gene encoding a type I myosin (*myo1⁺*; D. P. Mulvihill and J. S. Hyams, in preparation) and two genes encoding type II myosins, *myo2⁺* and *myp2⁺* (Bezanilla et al., 1997; also known as *myo22⁺* (May et al., 1998a) and *myo3⁺*; Motegi et al., 1997). In the present report we show that, as in *S. cerevisiae*, fission yeast possesses two type V myosins which

Table 2. Primers used in this study

Name	Sequence (5′-3′)	Engineered restriction site
c2D10-ApaI-up	CTTCGTGGGCCCGGAGGCTCTCACAG	*Apa*I
c2D10-AscI-up	GAAGGCGCGCCTGATTGACCGATGTTTC	*Asc*I
c2D10-AscI-down	ATCGGCGCGCCTGATGAGAACACAACAC	*Asc*I
c2D10-PstI-down	TGCTTTCCTGCAGTTACTATATGATGCTTTGA	*Pst*I
c2D10-start	AATTAAAGTCGACTATGAGTCATGCAAGATTATC	*Sal*I
c2D10-end	ATATGGATCCGGTGTAACGTTTAATGATACTTG	*Bam*HI
c1919-ApaI-up	ATTATAGGGCCCAAGGAAAGCTCACCGCA	*Apa*I
c1919-AscI-up	AACGGCGCGCCGTTTCTTCCTTCATAATTT	*Asc*I
c1919-AscI-down	AGTGGCGCGCCGGAAGCTTATTTGCAGCT	*Asc*I
c1919-PstI-down	GCTAACGCTGCAGGAGACTTCGTACTTCAAT	*Pst*I
c1919-start	AATTTCTGTCGACCATGACATCGGGGATTTATTAC	*Sal*I
c1919-end	TTAAAACGTCGACGGAAACTAAGGCCCAGCTCC	*Sal*I
myo51C-forward	TATTATCACTTGAAAATAATCATATATACGAAGAGCTTCGACTTTCAGAGTTGATAA-ACTTATTGGCTAAA-GCTACATTACGGATCCCCGGGTTAATTAA	None
myo51C-reverse	AATATAGTATTAATGAGTACTAATATAAATTAAAAATATTGATCGGGTGTAACGTTTA-ATGATACTTGATA-AAAAGCTTAGAATTCGAGCTCGTTTAAAC	None
myo52C-forward	TCACTGTAGGCAACGTAGCCGACAATGATGTACAGAACTCGAGCGACGAAGAAAA-TCAAGTACCAAATGG-TATTAAAGTTCGGATCCCCGGGTTAATTAA	None
myo52C-reverse	AGCTCCAAATTTTGAAAGTAAAACCCCTAATTAGGGAATAAATAAGTAGGCAGAGC-ACCTTGAAAAATAA-CTAGATATTAGAATTCGAGCTCGTTTAAAC	None

Engineered endonuclease restriction sites are underlined.

we designate *myo51*$^+$ (protein Myo51) and *myo52*$^+$ (protein Myo52). We show here that Myo51 and Myo52 are involved in distinct cellular functions in fission yeast cells. Myo52 is involved in cell wall deposition associated with both polarised cell growth and has a role in cytokinesis. Myo51 is involved in cytokinesis as a non-essential component of the CAR.

MATERIALS AND METHODS

Strain maintenance and genetics

Strains used in this study are listed in Table 1. Strain maintenance and genetic techniques were carried out as described (Alfa et al., 1993). Strains without plasmids were grown on supplemented yeast extract (YES) medium at 25°C unless otherwise specified. Strains carrying plasmids were grown on appropriately supplemented minimal medium (MM) containing 7.5 μg/ml thiamine and maintained at 25°C unless otherwise specified.

Identification and deletion of *myo51*$^+$ and *myo52*$^+$

myo51$^+$ and *myo52*$^+$ were identified by searching the Sanger Centre Fission Yeast Genome Sequencing Project (www.sanger.ac.uk/Projects/S_pombe) for proteins containing the myosin GESGAGKT motif. *myo51*$^+$ and *myo52*$^+$ were found to reside on cosmids c2D10 (SPBC2D10) and c1919 (SPCC1919), respectively. Both genes are retrievable from the EMBL database (*myo51*$^+$ accession number AL031788; *myo52*$^+$ accession number AL035075). Approximately 80% of the *myo51*$^+$ and 85% of the *myo52*$^+$ coding sequence were deleted in the null mutants. This was performed by constructing a cassette which consisted of the *ura4*$^+$ gene flanked by sequences upstream and downstream of the gene targeted for deletion. To delete *myo51*$^+$ the upstream sequence was obtained by PCR of wild-type genomic DNA using the primers c2D10-ApaI-up and c2D10-AscI-up (Table 2) whereas the downstream sequence was obtained using the primers c2D10-AscI-down and c2D10-PstI-down. The upstream and downstream PCR products, each 1 kb in size, were sequentially cloned into the vector pSL1180 (a kind gift from T. Chappell) as an *Apa*/*Asc*I and an *Asc*I/*Pst*I fragment, respectively. This was then used to insert the *ura4*$^+$ gene into the *Asc*I restriction site that lies between the *myo51*$^+$ sequences. The same strategy was used to construct the *myo52*$^+$ integration cassette but using primers specific for the *myo52*$^+$ locus (c1919-ApaI-up, c1919-AscI-up, c1919-AscI-down and c1919-PstI-down). The *myo51*$^+$ and *myo52*$^+$ integration cassettes were then cut from the vector and separately transformed into the diploid *leu1-32 ura4-D18 ade6-210 h*$^-$ / *leu1-32 ura4-D18 ade6-216 h*$^+$ by the lithium acetate method (Alfa et al., 1993). Homologous recombination was selected by growing transformants in supplemented minimal medium lacking uracil. Correct integration of the deletion cassettes was confirmed by Southern blot analysis of genomic DNA isolated from transformants.

Overexpression of *myo51*$^+$ and *myo52*$^+$

The *myo51*$^+$ gene was cloned by PCR of wild-type genomic DNA using the primers c2D10-start and c2D10-end (Table 2). The forward primer (c2D10-start) contained an engineered *Sal*I restriction site immediately upstream of the *myo51*$^+$ start codon and the reverse primer (c2D10-end) contained an engineered *Bam*HI restriction site to facilitate cloning into plasmids. This produced a 4.5 kb PCR product which was subsequently cloned into the *Sal*I and *Bam*HI unique restriction sites of pREP41 to yield pREP41-*myo51*$^+$ plasmid. Similarly, the *myo52*$^+$ gene was cloned by PCR using the primers c1919-start and c1919-end both of which contained an engineered *Sal*I restriction site. A 4.8 kb *myo52*$^+$ PCR product was obtained and cloned into the *Sal*I restriction site of pREP41 to yield pREP41-*myo52*$^+$ plasmid. The lithium acetate method (Alfa et al., 1993) was used to separately transform pREP41-*myo51*$^+$ and pREP41-*myo52*$^+$ plasmids into a wild-type leucine auxotroph (*leu1-32 ura4-D18 ade6-210 h*$^-$) and transformants were selected on supplemented minimal medium containing thiamine. To overexpress *myo51*$^+$ and *myo52*$^+$, transformants containing either pREP41-*myo51*$^+$ or pREP41-*myo52*$^+$ were induced by growing them in minimal medium free of thiamine for 24 hours at 29°C.

Tagging *myo51*$^+$ and *myo52*$^+$

Myo51 and Myo52 were tagged in two ways. First, an *Nde*I/*Nde*I GFP fragment from pGEM-GFP (Craven et al., 1998) was inserted in frame into the *Nde*I cloning site of the plasmids pREP41-*myo51*$^+$ and pREP41-*myo52*$^+$ to create pREP41GFP-*myo51*$^+$ and pREP41GFP-*myo52*$^+$, respectively. This enabled the production of GFP-Myo51 and GFP-Myo52 fusion proteins with GFP tagged at the N terminus. Second, *myo51*$^+$ and *myo52*$^+$ were fused with GFP at their chromosomal loci using the method described previously (Bähler et al., 1998) to create the strains *myo51-gc* and *myo52-gc* (Table 1) with Myo51 and Myo52, respectively, tagged at their C terminus and under the control of the native promoter. The GFP integration module to tag *myo51*$^+$ was amplified by PCR using the primers myo51C-forward and myo51C-reverse (Table 2) and the template pFA6a-GFP(S65T)-kanMX6. The same strategy was used to tag *myo52*$^+$ but using the primers myo52C-forward and myo52C-reverse. The GFP integration modules were then separately transformed into a wild-type strain (*leu1-32 ura4-D18 ade6-210 h*$^-$) by the lithium acetate method (Bähler et al., 1998). Integrants were selected on supplemented yeast extract medium containing 100 mg/l Geneticin (Life Technologies).

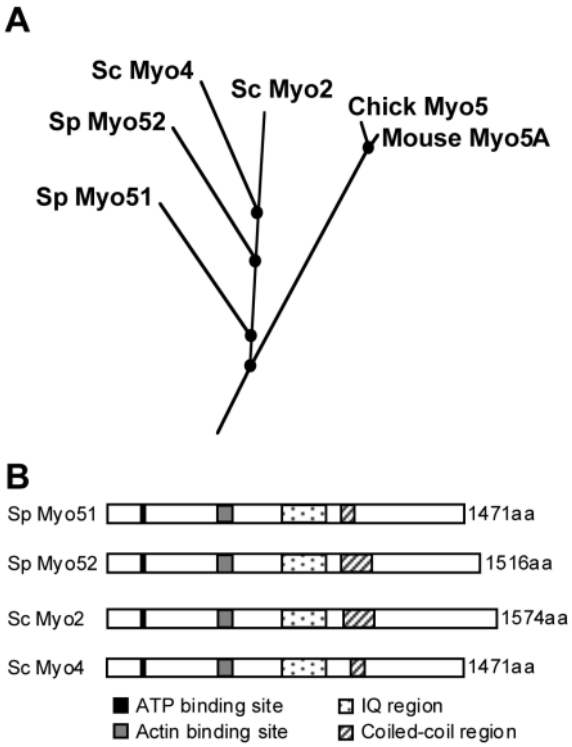

Fig. 1. (A) Unrooted phylogenetic tree showing the relationship of the four yeast type V myosins to each other and to representative vertebrate type V myosins. Closed circles at branch points represent >95% bootstrap values. Sp=*S. pombe*, Sc=*S. cerevisiae*. Accession numbers are as follows: chick Myo5 (MysD) Swiss-Prot Q02440; Mouse Myo5A Swiss-Prot Q99104; Myo2 Swiss-Prot P19524; Myo4 Swiss-Prot P32492; Myo51 EMBL AL031788; Myo52 EMBL AL035075. (B) Diagram summarising the structure of the two fission yeast and two budding yeast type V myosins. Myo52 is structurally similar to Myo2 and Myo51 is structurally similar to Myo4.

Correct integration of the GFP module at the *myo51*$^+$ chromosomal locus was confirmed by PCR using the primers c2D10-start and c2D10-end whereas c1919-start and c1919-end were used to confirm the correct integration of the GFP module at the *myo52*$^+$ locus.

Fluorescence microscopy

DAPI (4′-6-diamidino-2-phenylindole) and Calcofluor staining and indirect immuno-fluorescence microscopy of formaldehyde-glutaraldehyde fixed cells was carried out as described (Alfa et al., 1993). To visualise Myo51 and Myo52 tagged with GFP, exponentially growing cells expressing the fusion protein were fixed with cold methanol (–70°C) for 8 minutes. Fixed samples were washed twice in PBS and resuspended in PBS before mounting with DAPI. To observe actin and Mok1, cells were fixed with formaldehyde-glutaraldehyde and probed with either N-350 monoclonal anti-actin antibody (Amersham) or anti-Mok1 polyclonal antibody (a kind gift from T. Toda) at 1:100 dilution. Rhodamine-conjugated anti-mouse or anti-rabbit (Sigma) was used as the secondary antibody at 1:100. Microscopy was performed using a Zeiss Axiophot photomicroscope with a 63× 1.25 NA Neofluor objective. Images were captured with a Hamamatsu digital camera and analysed using OpenLab software (Improvision Ltd, Coventry, UK).

Treatment with actin- and microtubule-depolymerising drugs

To show that the localisation of Myo51 and Myo52 were dependent on an intact actin cytoskeleton, fission yeast cells were treated for 5 minutes with 10 μM final concentration of Latrunculin B (Molecular Probes) (Katayama et al., 1999). To depolymerise microtubules cells were treated for 1 hour with 300 μg/ml final concentration of thiabendazole (Sigma) (Petersen et al., 1998). In both cases DMSO alone was used as the control.

Zymolyase cell wall digestion assay

To test the sensitivity of fission yeast cell walls to the enzyme Zymolyase, cells were grown in YES at 25°C to exponential phase and centrifuged to obtain a pellet of 10×10^6 cells. The pellets were resuspended in 1 ml of 300 μg/ml Zymolyase-20T (ICN) solution and incubated at room temperature. The change in optical density (OD_{600}) with time was used as a measure of the progress of cell wall digestion. The viability of Zymolyase-digested cells was also quantified by plating out 1000 cells at intervals onto supplemented yeast extract medium in duplicate. Plates were incubated at 25°C for four days before they were scored for colonies.

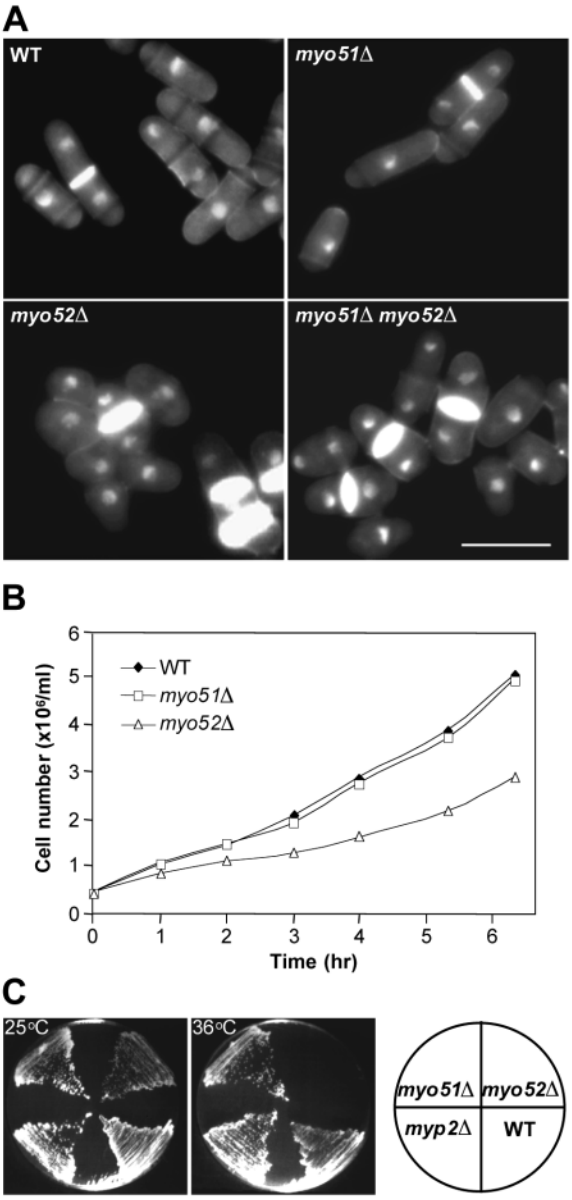

Fig. 2. Phenotypes and growth properties of *myo51Δ* and *myo52Δ*. (A) *myo51Δ* shows normal morphology whilst *myo52Δ* shows defects in cell polarity and cytokinesis. Cells lacking Myo52 are shorter and broader than the wild-type strain and a small proportion of cells (~1%) have multiple septa. The double deletion *myo51Δ* and *myo52Δ* is viable and resembles *myo52Δ*. Bar, 10 μm. (B) Growth curve. At 29°C *myo52Δ* grows slower than *myo51Δ* which has a growth rate comparable to wild type. (C) *myo52Δ* cells show no growth at 36°C. By contrast *myo51Δ* and cells deleted for the type II myosin *myp2*$^+$ (Mulvihill et al., 2000) grow normally at all temperatures.

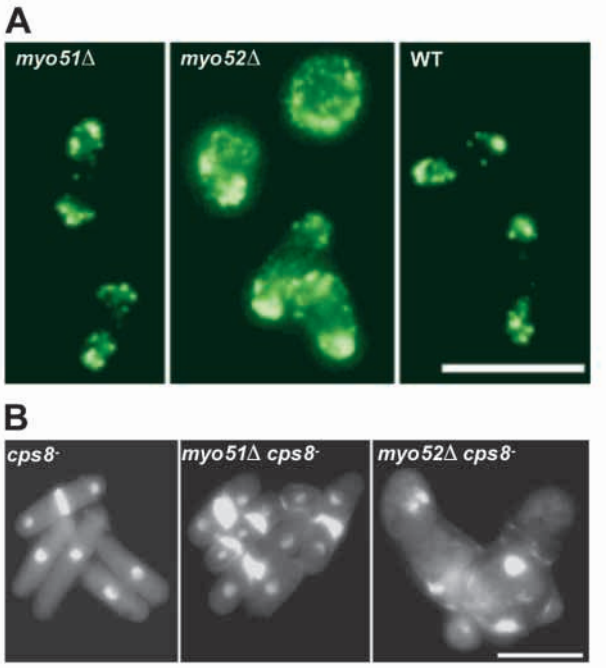

Fig. 3. Actin organisation in *myo51Δ* and *myo52Δ*. (A) Actin is partially depolarised in *myo52Δ* cells, completely so in round cells. Wild-type cells and *myo51Δ* are shown for comparison. Bar, 10 μm. (B) *myo51Δ* and *myo52Δ* show distinct phenotypes in combination with the actin mutant *cps8*$^-$. *myo51Δ cps8*$^-$ double mutant is viable at the permissive temperature whereas *myo52Δ cps8*$^-$ formed micro colonies of severely distorted cells. Bar, 10 μm.

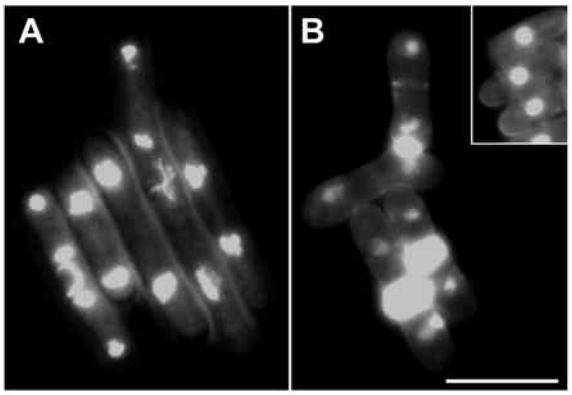

Fig. 4. Overproduction of Myo51 results in defects in cytokinesis whereas overproduction Myo52 results in defects in growth and cytokinesis. Wild-type cells containing the plasmids pREP41-*myo51*$^{+}$ or pREP41-*myo52*$^{+}$ were grown for 24 hours in the absence of thiamine (promoter on). Myo51 cells became elongated with aberrant septa whereas Myo52 cells were branched and contained multiple septa. Control cells grown in the presence of thiamine (promoter off) are shown in the insert. Bar, 10 μm.

Immunoblotting of Mok1

Wild-type and *myo52Δ* cells were grown to exponential phase in YES medium at 25°C. Cells were harvested and total protein extracts were prepared by smashing cells with 425-600 μm diameter glass beads (Sigma) using 4 M urea protein extraction buffer containing 5 mM Tris-HCl (pH 6.8). The protein extracts were run on a 7% SDS polyacrylamide gel after which they were transferred onto an Immobilon-P membrane (Millipore) using a Milliblot semi-dry blotting apparatus (Millipore). The membrane was then blocked with 1% Marvel in 1× PBS for 1 hour at room temperature after which a 1:1000 dilution of anti-Mok1 polyclonal antibody (Katayama et al., 1999; a kind gift from T. Toda) in 1% Marvel in 1× PBS was used to probe the membrane at 4°C overnight. The membrane was then treated to three 10 minute washes with 20 ml 1× PBS and incubated in a 1:1000 dilution of anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) in 1% Marvel in 1× PBS. Incubation with secondary antibody was carried out at room temperature for 4 hours after which the membrane was washed three times with 1× PBS as described previously. The membrane was then developed with the ECL chemiluminescent kit (Amersham) and protein bands were detected by exposing Bio Max film (Kodak) to the membrane. As a protein loading control the TAT1 anti-tubulin antibody (a kind gift from K. Gull) was used to probe the membrane as described above and detected with the anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma).

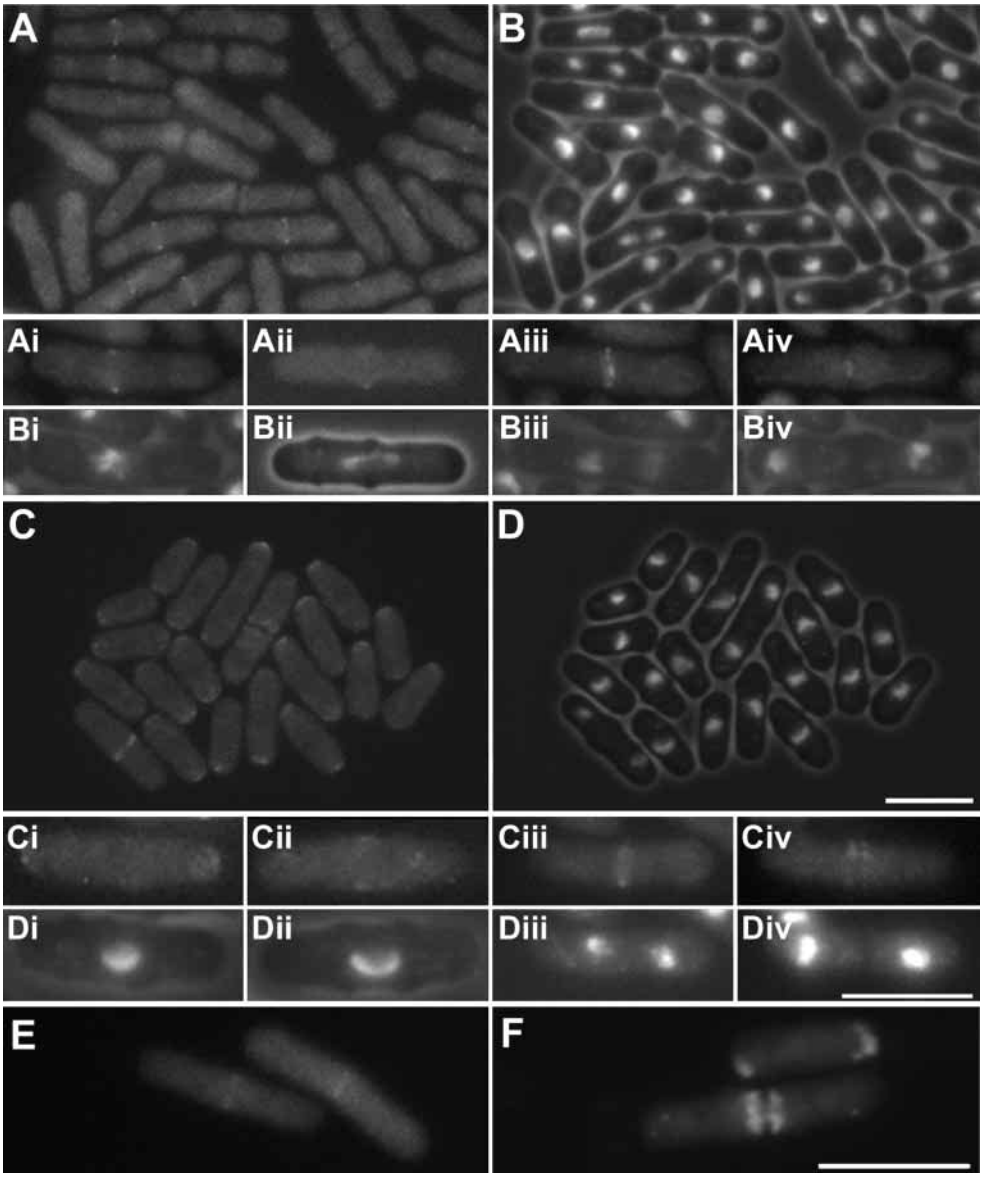

Fig. 5. Localization of Myo51 and Myo52. (A) *myo51-gc* cells with their corresponding DAPI images (B). Wild-type cells expressing GFP-Myo51 are shown in E. Myo51 is present at the CAR. Myo51 is detectable at the cell equator prior to any change in the morphology of the nucleus (Ai). At late anaphase the ring contracts (Aiv). (C) *myo52-gc* cells with their corresponding DAPI images (D). Wild-type cells expressing GFP-Myo52 are shown in F. Myo52 forms a cap at the poles of interphase cells, as a single equatorial band at the metaphase-anaphase transition (Cii) which intensifies as cells initiate septation (Ciii). A double band is seen in septated cells (Civ). Bar, 10 μm.

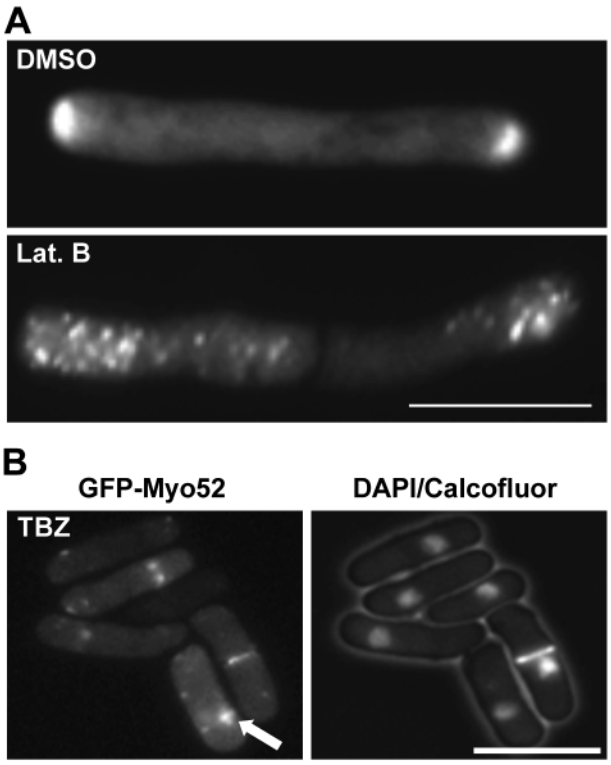

Fig. 6. (A) Localization of Myo52 is actin-dependent. Exposure of *cdc25-22* cells expressing GFP-Myo52 from a plasmid to 10 μM latrunculin dispersed the polar cap of Myo52 into small dots. Under these conditions actin patches are completely depolymerised. Bar, 10 μm. (B) Treatment of cells expressing GFP-Myo52 with TBZ results in the Myo52 cap being displaced from the tip (arrow). Equatorial Myo52 is not affected by TBZ. Bar, 10 μm.

RESULTS

Fission yeast has two type V myosins

Two fission yeast class V myosins were identified by searching the Sanger Centre Fission Yeast Genetic Database (www.sanger.ac.uk/Projects/S_pombe) for proteins containing the GESGAKT motif that is found within the P-loop of all myosins. *myo51*$^{+}$ (accession number EMBL AL031788) is located on chromosome II and encodes a protein of 1471 amino acids with a predicted molecular mass of 167,657 and a predicted pI=9.1. *myo52*$^{+}$ (accession number EMBL AL035075) is located on chromosome III and encodes a protein of 1516 amino acids with a predicted molecular mass of 175,155, and a predicted pI=6.2. The genomic sequence of *myo51*$^{+}$ contains a 55 base pair (bp) intron between nucleotides 30-86 and a 39 bp intron between nucleotides 4006-4046. *myo52*$^{+}$ also contains two introns; the first occurs at an almost identical position to that of *myo51*$^{+}$ (nucleotides 30-90), the second between nucleotides 197-272. The classification of these myosins was determined by comparison of their head domains with representative sequences from different myosin classes using PHYLIP software (http://evolution.genetics.washington.edu/phylip.html). This analysis confirmed that Myo51 and Myo52 lie on the same branch of the myosin V tree as the corresponding proteins in *Saccharomyces cerevisiae*, Myo2p and Myo4p (Fig. 1A). Both Myo51 and Myo52 consist of a head domain, which contains the ATP- and actin-binding sites, a neck region that contains the typical six IQ repeats (although not all of these may be functional) and a tail that is shorter than those of vertebrate type V myosins but similar to MYO2 and MYO4 (Johnston et al., 1991; Haarer et al., 1994). The tails of both Myo51 and Myo52 both contain regions predicted to form coiled-coils; a short sequence of 45 amino acids in the case of Myo51 and a longer region (143 amino acids) in Myo52. Interestingly, budding yeast Myo4 shows a similar coiled-coil structure to Myo51 (these two myosins in fact consist of the same number of amino acids) and Myo2 has a similar coiled-coil prediction to Myo52. These features are summarised in Fig. 1B.

Deletion and overproduction of Myo51 and Myo52 gives distinct phenotypes

In order to approach the function of the two fission yeast type V myosins we deleted both genes from their respective chromosomes. We independently replaced 80% of the coding sequence of *myo51*$^{+}$ and 85% of *myo52*$^{+}$ with the *ura4*$^{+}$ selectable marker in a diploid strain and confirmed that we had deleted the correct genes by Southern blotting. At 29°C the morphology and growth rate of cells lacking Myo51 were indistinguishable from an isogenic wild-type strain (Fig. 2A,B). At this temperature *myo52Δ* cells grew more slowly (Fig. 2B) and had an altered morphology, being shorter and

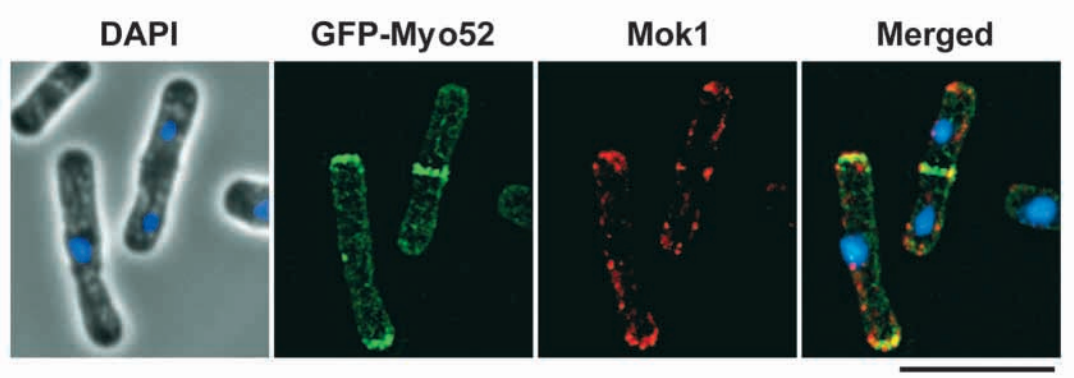

Fig. 7. Myo52 and Mok1 partially colocalise. Wild-type cells expressing GFP-Myo52 were processed for immunofluorescence microscopy using an anti-Mok1 antiserum. The merged images show a partial colocalisation of the two signals at the poles and septum. Deconvolved images. Bar, 10 μm.

broader with a septation index of 20%, twice that of wild type (Fig. 2A). *myo52Δ* cells showed extremely slow growth at 36°C (Fig. 2C). The morphology and growth defects of *myo52Δ* were not reversed by the inclusion of 1 M sorbitol in the growth medium to provide osmotic stability. The double mutant *myo51Δ myo52Δ* was viable and phenotypically similar to *myo52Δ* (Fig. 2A). Loss of cell shape in *myo52Δ* was accompanied by the depolarisation of the actin cytoskeleton whereas no change in actin organisation could be detected in *myo51Δ* (Fig. 3A). *myo51Δ* and *myo52Δ* showed distinct interactions with the temperature-sensitive actin mutant *cps8*$^{-}$ (Ishiguro and Kobayashi, 1996). The double mutant *myo52Δ cps8*$^{-}$ was extremely sick even at the permissive temperature and formed only micro colonies consisting of severely deformed cells. *myo51Δ cps8*$^{-}$ cells were viable at 25°C but had marked cytokinetic defects (Fig. 3B).

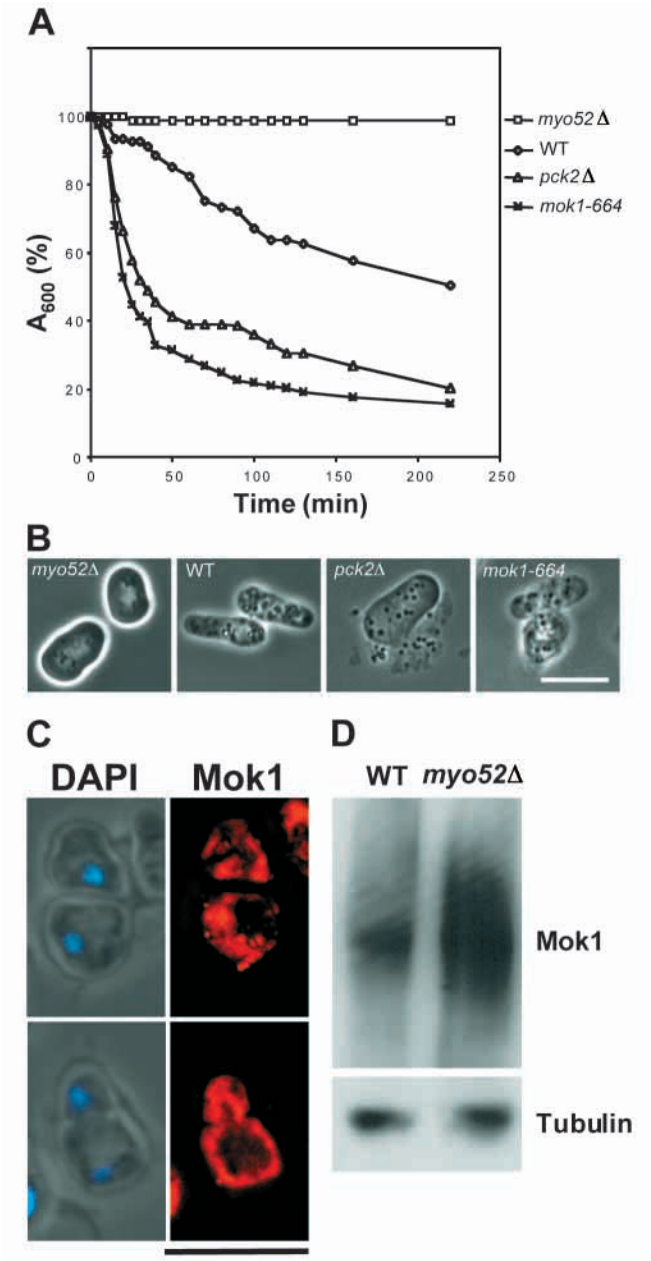

Fig. 8. *myo52Δ* cells are resistant to the cell wall degrading enzyme Zymolyase. (A) In comparison to wild type, *pck2Δ* and *mok1-664* cells, *myo52Δ* cells show resistance to digestion by Zymolyase as judged by the change in optical density of cultures treated with the enzyme. (B) Phase contrast micrographs showing the change in cell morphology following 4 hour digestion in Zymolyase. Note the integrity of the phase ring around the *myo52Δ* cells reflecting the intact cell wall. Bar, 10 μm. Mok1 is delocalised in *myo52Δ* cells (C; bar, 10 μm) and upregulated (D). By immunoblotting, *myo52Δ* cells contain a higher content of Mok1 than wild-type cells.The same samples were blotted with an anti tubulin antibody as a loading control.

To assess the effect of overproduction of Myo51 and Myo52, each gene was transformed independently into a wild-type strain in the plasmid pREP41 under the control of the thiamine-repressible *nmt1*$^{+}$ promoter. In the absence of thiamine, elevated levels of both Myo51 and Myo52 were toxic but the resulting phenotypes were distinct. Whereas Myo51 overproduction resulted in elongated cells with wispy, mis-oriented septal material (Fig. 4A), cells overproducing Myo52 were less elongated and branched due to the failure of septa to properly cleave (Fig. 4B).

Myo52 localizes to both cell poles and the septum; Myo51 is a component of the cytokinetic actin ring

To investigate the localization of Myo51 and Myo52 through the cell cycle we tagged both proteins in two distinct ways. First, the N terminus of each gene was fused to green fluorescent protein (GFP) in an appropriately modified pREP41 vector which was then transformed into *myo51Δ*, *myo52Δ* or wild-type cells. The *nmt1*$^{+}$ promoter is somewhat leaky and cells grown in the presence of thiamine expressed a level of GFP-Myo52 that fortuitously rescued both the temperature sensitivity and the morphology defect of the *myo52Δ* strain and a level of GFP-Myo51 that rescued the synthetic lethality of *myo51Δ cps8*$^{-}$ (see above). Thus, both fusion proteins were functional. Second, we constructed the strains *myo51-gc* and *myo52-gc* in which the 3′ end of the chromosomal copy of *myo51*$^{+}$ and *myo52*$^{+}$ was fused with GFP. These integrant strains, in which the tagged gene is under the control of its own promoter, retained the wild-type fission yeast morphology and growth rates.

Whether the protein was tagged at the N- or C terminus and whether expressed from its own or a heterologous promoter, Myo51 and Myo52 showed distinct patterns of localization. Images from the two integrant strains are shown in Fig. 5A-D. Wild-type cells expressing low levels of GFP-tagged *myo51*$^{+}$ and *myo52*$^{+}$ from plasmids are shown for comparison in Fig. 5E-F. Whereas Myo51 showed no obvious localisation in interphase cells (Fig. 5A,B), Myo52 formed a cap at the growing tips (Fig. 5C,D). In deconvolved images this could be seen to be composed of small, vesicle-like structures (Fig. 7). Myo52 tip staining was asymmetric with more Myo52 at one cell pole than the other. For most of the G_2 phase of the cell cycle fission yeast cells exhibit biased polar growth (Mitchison and Nurse, 1985), and this is reflected in the asymmetric distribution of actin at the cell poles (Marks and Hyams, 1985). When cells expressing GFP-Myo52 were stained with actin antibody, the Myo52-rich cell pole was found to be the actin-rich pole (the ‘old’ cell end; Mitchison and Nurse, 1985; data not shown). At the equator, Myo52 formed a ring which anticipated the formation of the septum (Fig. 5C, Cii and Ciii).

In cells which had already formed a septum a double band of Myo52 fluorescence was observed (Fig. 5C and Civ). Myo51 was also detected at the equator during cell division as a ring that was seen to contract (Fig. 5A and Ai-Aiv). The myosin II light chain Cdc4 is essential for the formation of the cytokinetic actin ring (CAR; Naqvi et al., 1999). We therefore made the strain *myo51-gc cdc4-8* and examined whether Myo51 rings were formed at 36°C. No rings were detected (data not shown) and hence, Myo51 appears to be a component of the CAR. The localisation of Myo51 and Myo52 at the cell equator is not coincident. Myo51 is detected as a band over the nucleus prior to any change in nuclear morphology as judged by DAPI staining (Fig. 5Ai). The equatorial band of Myo52 on the other hand appears coincident with the metaphase-anaphase transition (Fig. 5Cii). Finally, we asked whether the localization of Myo51 and Myo52 was dependent on the presence of the other myosin V by expressing each as a GFP fusion in the opposite null strain. Myo51 was found to localise normally in *myo52Δ*, Myo52 localised normally in *myo51Δ* and both localised normally in the double deletion *myo51Δ myo52Δ* (data not shown).

The localization of Myo51 and Myo52 is dependent upon an intact actin cytoskeleton

To further investigate the interaction of Myo51 and Myo52 with actin, wild-type or *cdc25-22* cells (which are longer than wild type) producing GFP-Myo51 or GFP-Myo52 were exposed to latrunculin B (lat B) at a concentration that completely depolymerises the actin cytoskeleton. In the presence of lat B Myo52 was dispersed into a number of small dots which may be vesicles (Fig. 6A). This was reversed upon removal of the drug. A similar effect was seen with cytochalasin A and in *cps8*$^{-}$ at the restrictive temperature (data not shown). In cells expressing GFP-Myo52 (in which the protein is moderately overexpressed), Myo52 was also reversibly displaced from the cell poles by a concentration of thiabendazole (TBZ) that completely abolishes the cytoplasmic microtubules network in *S. pombe* (Petersen et al., 1998) although in this case the polar cap of Myo52 staining did not disperse but appeared to be dislodged from its polar location. TBZ did not disturb the equatorial localisation of Myo52 (Fig. 6B). The polar displacement of Myo52 by TBZ was not seen in *myo52-gc* in which the protein is produced at native levels. TBZ had no effect on Myo51 but Myo51 rings were undetectable after exposure to lat B (data not shown).

Myo52 is involved in cell wall deposition

We were struck by the similarity of the Myo52 staining patterns we observed with those previously shown for the enzyme α-glucan synthase, especially as this localization is also actin-dependent (Katayama et al., 1999). α-glucan synthase, encoded by the gene *mok1*$^{+}$ (Katayama et al., 1999; also known as *ags1*$^{+}$, Hochstenbach et al., 1998), is involved in the deposition of α-glucan, one of the two major structural components of the fission yeast cell wall. Myo52 and Mok1 colocalised at the poles and, to a small extent, also at the equator (Fig. 7).

If Myo52 is involved in Mok1 localization it might be expected that the integrity of the cell wall would be altered in a *myo52Δ* strain. To test this we compared the sensitivity of *myo52Δ* to the enzyme zymolyase to that of wild-type cells and two mutants having well characterised sensitivity to this enzyme, *mok1-664* and *pck2*Δ (Katayama et al., 1999). As judged by the change in optical density of cultures exposed to zymolyase, *myo52Δ* was significantly more resistant to digestion than wild type (Fig. 8A) and this was confirmed by the morphology of the cells (Fig. 8B) and viability counts (not shown). *myo51Δ* showed wild-type sensitivity to Zymolyase. Mok1 was delocalised in *myo52Δ* (Fig. 8C) and also apparently upregulated, western blotting of total protein extracts showing a Mok1 band that was at least two-fold more intense than that seen in wild-type extracts (Fig. 8D).

DISCUSSION

Although the sequencing of the fission yeast genome is still incomplete, it appears that, like budding yeast, fission yeast possesses two genes encoding type V myosins. There was no a priori reason to assume a correspondence between the inventory of myosin V genes in the two yeasts since myosin II is represented by two genes in *S. pombe* (Bezanilla et al., 1997; Kitayama et al., 1997; May et al., 1997; Motegi et al., 1997; reviewed by May et al., 1998a) and only one in *S. cerevisiae* (Watts et al., 1987; Sweeney et al., 1990) whilst budding yeast possesses two genes encoding type I myosins, *MYO3* and *MYO5* (Goodson and Spudich, 1995; Goodson et al., 1996) and, as yet, only a single member of this class has been identified in *S. pombe* (DPM and JSH, in preparation). As originally predicted (Cheney et al., 1993), several lines of evidence point to the yeast type V myosins as defining a distinct subgroup within this class of unconventional myosins, a situation we have suggested also exists for the yeast type II myosins (May et al., 1998a,b). The most compelling evidence is that phylogenetic analysis of the head domain of all four yeast type V myosins places this group on a separate branch of the myosin family tree (see also Schott et al., 1999). The tails of these myosins are shorter than those of metazoans (Espreafico et al., 1992) a difference accounted almost entirely by a reduction in the length of the predicted coiled-coil domain. Structurally, Myo52 more closely resembles budding yeast Myo2p than it does Myo4p which more closely resembles Myo51. In budding yeast, Myo2p is implicated in the polarised delivery of membrane-bound organelles (Johnston et al., 1991; Govindan et al., 1995; Hill et al., 1996; Santos and Snyder, 1997; Catlett and Weisman, 1998; Pruyne et al., 1998; Schott et al., 1999; Catlett et al., 2000) and is involved in the orientation of the mitotic spindle (Yin et al., 2000). Myo4p on the other hand is required for mating type switching (Bobola et al., 1996; Jansen et al., 1996; Long et al., 1997; Takizawa et al., 1997; Bertrand et al., 1998). Both fission yeast type V myosins contain six IQ motifs although not all may be functional. The distribution of calmodulin in fission yeast (Moser et al., 1997) parallels that of Myo52 (although, unlike calmodulin, Myo52 is not found at the spindle poles) and it is likely that, at least in part, this reflects its association with Myo52. The phenotypes of strains lacking or overproducing Myo51 and Myo52 are distinct and the two proteins localise differently through the fission yeast cell cycle. Taken together with the fact that the *myo51Δ myo52Δ* double mutant is viable, it appears that two fission yeast type V myosins have distinct, non-overlapping functions. Myo52 was localised to the poles

of growing cells and to the septum in cells undergoing cytokinesis. During cell growth, the faster growing pole (the old end; Mitchison and Nurse, 1985), as with actin (Marks and Hyams, 1985), has more Myo52 than the slower growing new end. The actin-rich region at both poles is more extensive than that occupied by Myo52 and, hence, the two proteins do not precisely colocalise. We have shown previously that tip growth and septation in *S. pombe* are sensitive the myosin ATPase inhibitor BDM and the localisation of Myo52 to these sites provides a possible explanation for this inhibition (May et al., 1998b). Both latrunculin B and cytochalasin A caused the polar cap of Myo52 to disperse into a number of dots which may represent small vesicles and in deconvolved images the cap is seen to have a punctate substructure. The Myo52 cap is, hence, reminiscent of the Spitzenkörper, the vesicle-rich body found at the growing tips of filamentous fungi (Seiler et al., 1997; Lehmler et al., 1997). Electron microscopy has confirmed the presence of vesicles at the cell poles in fission yeast (Kanbe et al., 1989). The integrity of the Spitzenkörper is dependent on an intact microtubule cytoskeleton and, in particular, the function of the microtubule motor protein, kinesin (Seiler et al., 1997; Lehmler et al., 1997). Some type V myosins are physically associated with a kinesin-related motor (Huang et al., 1999; Beningo et al., 2000) but whether Myo52 falls into this category awaits further investigation. The localisation of Myo52 at the cell tips (but not at the equator) was microtubule-dependent but only in cells in which GFP-Myo2 was modestly overexpressed. In this case the Myo52 cap, but not equatorial Myo52, was displaced by TBZ, indicating either that excess protein targets correctly but does not anchor. Myo51 rings were undetectable following exposure to latrunculin B. The association of Myo51 with the CAR is therefore different to that of the type II myosin, Myo2 which retains its ring morphology in the absence of actin (Naqvi et al., 1999).

A single ring of Myo52 appears at the incipient division site prior to the appearance of the primary septum; subsequently this becomes a double ring as the secondary septum is laid down. How the Myo52 ring is positioned is not clear although it is more likely to be a function of the CAR, which forms early in mitosis (Marks and Hyams, 1985), than the equatorial ring of microtubules that forms late in spindle elongation in fission yeast (Pichova et al., 1995). Additionally, equatorial Myo52 is delocalised by anti-actin but not anti-tubulin drugs. Importantly, the Myo52 ring does not contract. Myo52 is therefore not, like the two type II myosins, Myo2 and Myp2 (Kitayama et al., 1997; Bezanilla et al., 1997; Mulvihill et al., 2000) nor, indeed, Myo51, a component of the CAR. Rather, it appears to be associated with the septum that forms centripetally as the actin ring contracts, perhaps associated with the small vesicles that accompany septum ingrowth (Kanbe et al., 1989). *myo52Δ* has a septation index that is about twofold higher than that in wild-type cells, suggesting that, in the absence of Myo52, septa form more slowly or fail to cleave properly. Thus, Myo52 is clearly not responsible for the bulk delivery of septal components but possibly of some minor component that is essential for septum maturation and cleavage. Surprisingly, Myo51 appears to be a component of the CAR, as far as we are aware the first time a type V myosin has been shown to have a role in cytokinesis. This conclusion is based on the appearance of Myo51 at the cell equator at the same time as the CAR component Myo2, on the fact that the Myo51 ring reduces in diameter as mitosis proceeds and on the dependence of the Myo51 ring on CAR integrity (based on the abolition of Myo51 staining in *cdc4-8* at the restrictive temperature). The precise role of Myo51 in cytokinesis awaits further study.

The localization of Myo52 to sites of cell wall deposition (the cell poles and septum), regions that are also enriched in actin (Marks and Hyams, 1985), points to this myosin playing a role in the delivery or anchorage of some component of the cell wall synthesising machinery to these locations, a similar role to that played by Myo2p in budding yeast (Johnston et al., 1991; Govindan et al., 1995; Pruyne et al., 1998; Schott et al., 1999). The synthesis of cell wall α-glucan is specified by a family of α-glucan synthases (Hochstenbach et al., 1998; Katayama et al., 1999). Mok1 localises to the growing poles and septum in an actin-dependent manner and mutants in *mok1*$^+$ / *ags1*$^+$ result in major cell wall defects (Katayama et al., 1999). When cells expressing GFP-Myo52 were stained with an antibody to Mok1, Myo52 and Mok1 were seen to colocalise at the cell tips and, partially, at the septum but not elsewhere in the cell. In the absence of Myo52 Mok1 was delivered to the cell surface but inappropriately localised. Thus, Myo52 is not the motor that delivers Mok1 to the cells poles but appears to be essential for its precise localisation to sites of wall deposition. Deletion of Myo52 results in the upregulation of Mok1 and the formation of a cell wall that is substantially more resistant to the cell wall degrading enzyme zymolyase. A defect in cell wall production is consistent with the slow, depolarised growth phenotype of the Myo52 null strain.

Fission yeast is becoming an increasingly valuable model for illuminating the fundamental principles underlying cell polarity (Mata and Nurse, 1998) and cytokinesis (Le Goff et al., 1999). One of the key determinants of growth polarity and septation in *S. pombe* is actin which is concentrated at sites of cell wall deposition (Marks and Hyams, 1985). The identification of Myo51 and Myo52 adds an important new component to the inventory of proteins involved in both of these processes.

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