Identification of MYO4, a second class V myosin gene in yeast

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SUMMARY

We have isolated a fourth myosin gene (MYO4) in yeast (Saccharomyces cerevisiae). MYO4 encodes a ~170 kDa (1471 amino acid) class V myosin, using the classification devised by Cheney et al. (1993a; Cell Motil. Cytoskel. 24, 215-223); the motor domain is followed by a neck region containing six putative calmodulin-binding sites and a tail with a short potential ‘coiled-coil’ domain. A comparison with other myosins in GenBank reveals that Myo4 protein is most closely related to the yeast Myo2 protein, another class V myosin. Deletion of MYO4 produces no detectable phenotype, either alone or in conjunction with mutations in myo2 or other myosin genes, the actin gene, or secretory genes. However, overexpression of MYO4 or MYO2 results in several morphological abnormalities, including the formation of short strings of unseparated cells in diploid strains, or clusters of cells in haploid strains. Alterations of MYO4 or MYO2 indicate that neither the motor domains nor tails of these myosins are required to confer the overexpression phenotype, whereas the neck region may be required. Although this phenotype is similar to that seen upon MYO1 deletion, we provide evidence that the overexpression of Myo4p or Myo2p is not simply interfering with Myo1p function.

Key words: myosin, MYO4, yeast

INTRODUCTION

The family of identified myosins is growing. In addition to the extensively studied conventional myosin, which dimerizes via a long α-helical tail, there are an increasing number of myosins that resemble conventional myosin in the motor domain but not in the rest of the molecule. Cheney et al. (1993a) have divided the myosins into classes based on the amount of sequence similarity in the motor domains. So far, myosins from three of these classes have been identified in yeast: Myo1 protein (Myo1p) (Watts et al., 1987; Rodriguez and Paterson, 1990) is a member of the myosin II class (= conventional myosin), and like the Dictyostelium myosin II has been implicated in cytokinesis. Myo3p (Goodson et al., 1990) is a myosin I (‘minmyosin’) whose function is unknown (deletion of MYO3 has no discernible phenotypic effect). Myo2p (Johnston et al., 1991) is a myosin V; studies of the temperature-sensitive myo2-66 mutant have implicated Myo2p in polarized growth (i.e. the budding process in yeast). Myo5 V’s have three characteristic domains; a ‘head’ or motor domain, a ‘neck’ with six repeats that putatively bind calmodulin, and a long ‘tail’ containing stretch(es) predicted to form coiled coils. Here we report the identification of a fourth yeast myosin, Myo4p, which, like Myo2p, is a member of the myosin V class.

MATERIALS AND METHODS

Growth and handling of yeast

Yeast strains (Table 1) were grown routinely at room temperature (~22°C) in the rich liquid medium YM-P (Lillie and Pringle, 1980) or on YEPD plates (Sherman et al., 1986). Standard techniques were used for transformation (by lithium acetate) and genetic manipulations (Sherman et al., 1986). Selection for plasmids was maintained by growth in synthetic complete medium with uracil or leucine (SC-uracil, SC-leucine), or on the corresponding plates (essentially as described by Sherman et al., 1986). Stability tests for plasmids were performed by inoculating cells from selective medium into YM-P. Cultures were grown to stationary phase (~8 generations) and plated on YEPD (Sherman et al., 1986). Resulting colonies were tested for the presence or absence of plasmid by streaking on selective plates.

DNA manipulations

Standard procedures were used for DNA manipulations (Sambrook et al., 1989). DNA fragments were subcloned in pBluescript (Stratagene, La Jolla, CA) for sequencing by the dideoxy method (Sanger et al., 1977) as described by the Sequenase DNA Sequencing Kit (United States Biochemical, Cleveland, OH). In some cases, oligonucleotides were synthesized for use as internal sequencing primers. Both strands were sequenced in and near the coding region for MYO4, and sequencing was carried out across cloning junctions on at least one strand. In addition, a single strand of flanking DNA was sequenced as indicated in Fig. 1.

Isolation of the complete MYO4 gene

The ~5.5 kbp EcoRI fragment (see Fig. 1, line b) obtained from C. Davies was used as a probe to screen, by colony hybridization, a YCP50 plasmid-based yeast genomic library (Rose et al., 1987). Several hybridizing plasmids were obtained, whose inserts overlapped or encompassed the probe DNA as judged by restriction mapping and Southern blot analysis. These inserts were used for subcloning and DNA sequence analysis.
Mapping of the MYO4 gene

A 3.5 kbp Sall-BgII fragment (contained entirely within the MYO4 coding region) was used to probe a yeast chromosome blot (generously provided by K. Corrado) on Genescreen Plus (New England Nuclear, Boston, MA). The DNA probe was labeled with $^{32}$P dATP by random primer labeling (Feinberg and Vogelstein, 1983) and was hybridized to the chromosome blot as described by New England Nuclear. Results suggested that MYO4 was on chromosome I; examination of transcript maps of chromosome I (Diehl and Pringle, 1991) suggested that MYO4 might correspond to the transcribed region reported as FUN22 (function unknown). Comparison of downstream DNA sequence to sequence of the FUN20 region (accession no. X62577) confirmed this location of MYO4. (However, the direction of the MYO4 open reading frame is opposite the reported direction of transcription for FUN22 (Diehl and Pringle, 1991).)

Deletion of MYO4

The majority of the MYO4 gene was deleted as follows (refer to Fig. 1, line c): (i) an 881 bp SpeI fragment (which includes the C-terminal 25 codons of MYO4) was cloned into the SpeI-XbaI sites of pBlueScript KS$^+$; (ii) an ~320 bp KpnI-Sall fragment (containing codons 11-118 of MYO4) was cloned into the KpnI-SalI sites of the polynucleotid region of the plasmid from (i); (iii) the 1170 bp URA3-containing HindIII fragment from plasmid YEp24 was inserted in the HindIII site of the plasmid from (ii); the resulting plasmid was then digested at the KpnI and SacI sites of the polynucleotid region to generate an ~2.4 kbp fragment that contained the URA3 gene within MYO4 coding sequence. This KpnI-SacI fragment was used to transform the diploid yeast strain 22AB to Ura$^+$ (by one-step replacement (Rothstein, 1983) of one of the two copies of the MYO4 gene). Subsequent sporulation and tetrad dissection produced haploid segregants that lacked an intact MYO4 gene. DNA hybridization analysis of chromosomal DNA from the diploid heterozygote (myo4$^{+}$) and its haploid progeny indicated that the MYO4 gene deletion had occurred as expected.

Construction of full-length and altered MYO4-containing plasmids

The complete MYO4 gene was cloned into YEp351 (a LEU2-based yeast high-copy-number vector; Hill et al., 1986) by sequentially inserting a 4.7 kbp Sall-HindIII fragment from a library clone (YCpMYO4; the insert is indicated in Fig. 1, line a) into the Sall and HindIII sites in the polynucleotid region of YEp351, and then a 1.4 kbp Sall fragment into the Sall site, to form plasmid YEp351-MYO4 (Fig. 1, line b). Truncated forms of the MYO4 gene (Fig. 3B) were cloned into YEp351 as follows: (i) a 4.9 kbp BamHI-BgII fragment of YEp351-MYO4 was inserted into the BamHI site of YEp351 to create plasmid YEp351-MYO4(Bgl); (ii) a 3.6 kbp BamHI-Hpal fragment of YEp351-MYO4 was inserted into the BamHI-Smal sites of YEp351 to create plasmid YEp351-MYO4(Hpa); (iii) a 3.2 kbp XbaI-XbaI fragment of YEp351-MYO4 was inserted into the XbaI site of YEp351 to create plasmid YEp351-MYO4(Xba); (iv) a 2.4 kbp BamHI-EcoRV fragment of YEp351-MYO4 was inserted into the BamHI-Smal sites of YEp351 to create plasmid YEp351-MYO4(EcoRV). (In all cases, the upstream restriction site was in the polynucleotid region of YEp351 into which MYO4 had been inserted.)

It was also necessary to construct these plasmids with a different selectable marker in order to use them in a MYO1 disruptant (Table 4). Complete and truncated forms of MYO4 were cloned into the high-copy-number URA3-based vector YEp352 (Hill et al., 1986) as

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**Table 1. Saccharomyces cerevisiae strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>22AB</td>
<td>$\text{MATa ura3 trpl lys2 leu2 his3}$ a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{MATa ura3 trpl lys2 leu2 his3 MYO4}$ a</td>
<td></td>
</tr>
<tr>
<td>myo4A5</td>
<td>$\text{MATa ura3 trpl lys2 leu2 his3 MYO4}$ a</td>
<td>a</td>
</tr>
<tr>
<td>myo4A5-2A</td>
<td>$\text{MATa ura3 trpl lys2 leu2 his3 MYO4}$ a</td>
<td>a</td>
</tr>
<tr>
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<td>a</td>
</tr>
<tr>
<td>myo4A5-2C</td>
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<td>a</td>
</tr>
<tr>
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<td>$\text{MATa ura3 trpl lys2 leu2 his3 MYO4}$ a</td>
<td>a</td>
</tr>
<tr>
<td>(2A+2D)</td>
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<td>a</td>
</tr>
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<td>SLY55</td>
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<td>b</td>
</tr>
<tr>
<td>SLY74</td>
<td>$\text{MATa ura3 sec7-1}$ g</td>
<td>g</td>
</tr>
<tr>
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<td>$\text{MATa ura3 sec1-2}$ c</td>
<td>c</td>
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<td>DBY1999</td>
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<td>c</td>
</tr>
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<td>d</td>
</tr>
<tr>
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<td>d</td>
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<td>e</td>
</tr>
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<td>a,f</td>
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<tr>
<td>NY57</td>
<td>$\text{MATa ura3 sec9-4}$ g</td>
<td>g</td>
</tr>
<tr>
<td>NY61</td>
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</tr>
<tr>
<td>NY410</td>
<td>$\text{MATa ura3 sec8-9}$ g</td>
<td>g</td>
</tr>
<tr>
<td>NY432</td>
<td>$\text{MATa ura3 sec18-1}$ g</td>
<td>g</td>
</tr>
<tr>
<td>NY760</td>
<td>$\text{MATa ura3 sec7-1}$ g</td>
<td>g</td>
</tr>
</tbody>
</table>

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**Footnotes:**

*a* This study.

*b* S. Lillie, unpublished.

*c* Provided by D. Botstein.

*d* Provided by H. Goodson and J. Spudich.


*f* Segregant from cross of BGE1 by myo4A5-2D.

*g* Provided by P. Novick.
follows: Full-length MYO4 was moved as a 5.8 kbp BamHI-HindIII fragment from YEp351-MYO4 to YEp352 to create plasmid YEp352-MYO4. YEp352-MYO4(Bgl) and YEp352-MYO4(Hpa) were constructed essentially in the same manner as YEp351-MYO4(Bgl) and YEp351-MYO4(Hpa); YEp352-MYO4(Xba) was made by inserting the BamHI-HindIII fragment from YEp351-MYO4(Xba) into the BamHI and HindIII sites of YEp352; YEp352-MYO4(EcoRV) was made by inserting the BamHI-EcoRI fragment from YEp351-MYO4(EcoRV) into the BamHI and EcoRI sites of YEp352.

Deletions within the MYO4 gene (Fig. 3B) were created as follows: (i) YEp351-MYO4 was cut with NraI and NcoI, the ends were filled using Klenow fragment, and religated to create YEp351-MYO4(N, Nc). (ii) YEp352 was cut with BamHI in the polylinker region and ends were filled using Klenow fragment; then it was cut with HindIII in the polylinker region, and ligated to a ~2kb SspI-HindIII fragment from YEp351-MYO4 (the HindIII site is shown in Fig. 1, line d). Into the KpnI site in the polylinker region of this construct was inserted a 1 kbp KpnI fragment from YEp351-MYO4 (the upstream KpnI site was in the polylinker region of YEp351 into which MYO4 had originally been inserted), to create YEp352-MYO4(K, Ss). (iii) A 0.6 kbp XbaI-Xbal fragment of YEp351-MYO4 was inserted into the XhoI site in the polylinker region of YEp352. Into the KpnI site in the polylinker region of this construct was inserted a 1 kbp KpnI fragment (the same fragment as described in (ii)) from a YEp351-MYO4 whose BamHI site had first been destroyed (by cutting, filling with Klenow fragment, and religating). Finally, the BamHI site in the polylinker region of this construct was cut, filled using Klenow fragment, and religated to place the fragments of the MYO4 gene in frame, producing YEp352-MYO4(K, X, X).

Construction of full-length and altered MYO2-containing plasmids

YEpMYO2-40, which contains the complete MYO2 gene, was made by inserting the ~6 kbp ClaI fragment from pJP10-2B (Johnston et al., 1991) into the ClaI site of YEp102 (a high-copy-number yeast vector; Voytek et al., 1991). (The ClaI fragment includes vector sequence upstream of MYO2.) YEpmyo2 was constructed in the same manner as YEpMYO2-40, except that the ClaI fragment contained the temperature-sensitive myo2-66 allele (S. H. Lillie and S. S. Brown, unpublished data). YEpMYO2-42, which contains a truncated MYO2 gene, was made by inserting the ~4kbp EcoRI fragment from pJP10-2B into the EcoRI site of YEp352. (The EcoRI fragment includes vector sequence upstream of the MYO2 gene and truncates the MYO2 gene at codon 1116.)

Antibody production, western blotting and immunofluorescence

Fusion proteins were produced containing the tail portion of Myo4p (amino acids 975-1448; indicated in Fig. 2) fused to either trpE protein (see Haarer and Pringle, 1987) or β-galactosidase (Rüther and Müller-Hill, 1983). A 1.4 kbp SspI-SpeI fragment of the MYO4 gene was ligated into pATH1 cut with Smal and XhoI, or into pUR289 cut with EcoRV and XbaI. Polyclonal antibodies were raised in rabbits against these fusion proteins (in the manner described by Kim et al., 1991), and the antibody was affinity-purified against the heterologous fusion protein (in the manner described by Haarer and Pringle, 1987).

Western blots of yeast total protein were prepared: cells were grown in YM-P or SC-leucine (for strains harboring LEU2-based plasmids) to mid-log stage and were harvested by centrifugation, then broken in cold water by vortexing in the presence of 0.5 mm glass beads. Solubilization buffer was added to samples, which were then placed in a boiling water bath for 2 minutes and loaded on a 5% polyacrylamide gel with 3% stack). Gels were blotted to nitrocellulose at ~400 mAMP for 24 hours. Blots were probed with affinity-purified anti-Myo4p antibodies at ~1:60 dilution; horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was used at 1:250. Parallel staining of blots with anti-Myo2p antibody (S. H. Lillie and S. S. Brown, unpublished data) confirmed that the Myo4p antibodies do not crossreact with Myo2p.

Cell staining with anti-yeast actin antibodies, anti-yeast tubulin monoclonal antibody, Calcofluor (stains cell-wall chitin), and DAPI (stains DNA) were as described previously (Haarer et al., 1990, and references therein).

RESULTS

MYO4 sequence

A DNA fragment with sequence similarity to myosins was originally identified by Chris Davies (C. Davies, J. Trgovcich and C. A. Hutchison, unpublished data). We used this fragment (Fig. 1, line b) to obtain the complete MYO4 gene from a yeast genomic library. Sequence analysis revealed a predicted product of 1471 amino acids, with a calculated molecular mass of 169,342 and isoelectric point of 7.63.

We feel confident that we have determined the correct limits of the open reading frame, for the following reasons: There are stop codons in all three reading frames within 131 bp upstream and 119 bp downstream of the open reading frame. The SNC1 gene (Gerst et al., 1992) is found just 102 bp downstream, reading in the opposite direction. A search for splice recognition sequences revealed two TACTAAC sequences (the motif employed by yeast), however, these are in regions of conserved amino acid sequence (Fig. 2) and are therefore unlikely to indicate the existence of introns.

We have classified Myo4p as a class V myosin based on primary sequence and overall predicted structure (Fig. 3A). The other class V myosins that have been identified so far include yeast Myo2p (Johnston et al., 1991), a 190 kDa myosin from chick brain (p190; Larson et al., 1988, Espindola et al., 1992), and the protein encoded by the dilute locus in mice (Mecer et al., 1991). Dilute protein and p190 are probable homologs of one another, as they show about 90% identity throughout (Espaefico et al., 1992). A comparison of the primary amino acid sequence of Myo4p to that of other myosins showed that yeast Myo2p has the most closely related sequence, followed by dilute protein and p190 (Figs 2 and 3A). Other, non-class V, myosins are less similar to Myo4p in the head, and show no similarity in the tail. This can be seen in the dot matrix comparisons of yeast myosins shown in Fig. 4; while Myo1p (a conventional, class II myosin) resembles Myo4p only in the head, Myo2p shows similarity to Myo4p throughout.

Myo4p has several structural motifs that are characteristic of class V myosins. First, Myo4p has a ‘neck’, consisting of six 23-25 amino acid repeats, immediately downstream of the head (marked in Figs 2 and 3A and can be seen in Fig. 4, see legend). These repeats, referred to as ‘IQ domains’, each resemble sequences found in proteins containing calcium-insensitive calmodulin-binding sites (Cheny and Mosoeker, 1992). Class V myosins have six of these repeats, whereas other myosins have fewer such repeats (see the alignment in Espaefico et al., 1992). Espaefico et al. (1992) have mapped the calmodulin-binding sites of p190 to the neck by expressing fragments of p190 in bacteria.

Another characteristic that Myo4p shares with other class V myosins is a stretch predicted to be capable of coiled-coil formation (Lupas et al., 1991) in the first part of the ‘tail’ (Fig.
Fig. 2. Sequence comparison between Myo4p, Myo2p, dilute protein and p190. The PILEUP program (Devereux et al., 1984) was used for alignment. Identities between Myo4p and any of the other myosins are boxed. Brackets below the sequences mark the six IQ domains. Filled circles above Myo4p indicate locations of two TACTAAC potential splicing motifs (see text). Filled squares indicate the portion of Myo4p used for antibody production (amino acids 975-1448).
opposite to the reported direction of transcription.

Mapping MYO4

MYO4 was mapped to chromosome I by probing a yeast chromosome blot. We have determined that MYO4 is the same as FUN22, an open reading frame of unknown function on a transcript map of chromosome I (Diehl and Pringle, 1991), although we report an orientation of the coding region that is opposite to the reported direction of transcription.

Deletion of MYO4

The MYO4 gene was deleted by replacing the majority of the gene with the URA3 gene (Fig. 1, line c). This deletion removes all but 118 N-terminal and 25 C-terminal codons of MYO4. No phenotypic effects of the deletion were observed when diploid yeast were transformed with this construct and haploid segregants examined (cells containing the deletion could be identified as Ura+ progeny following tetrad analysis). We confirmed that the expected deletion had occurred by Southern blotting. (The Southern blots also revealed that there were no closely related, crosshybridizing sequences.) Segregants with the deletion grew at the same rate (Fig. 5) as the wild-type sister segregants when examined at several temperatures (16°C, 22°C, 36°C), and consistent with these results, showed no differences in the frequency of budded cells (Table 2, under “+control vector”). Furthermore, no morphological abnormalities were detected by light microscopy (Table 2, under “MYO4 plasmid”). MYO4 deletion strains were stained with anti-actin and anti-tubulin antibodies, with Calcofluor to localize chitin in the cell wall (Sloat et al., 1981), and with DAPI to examine the behavior of the nucleus during the cell cycle. Localization of all of these components appeared normal (for example, compare chitin localization and cell shape in Fig. 6A and B). MYO4 deletion strains also had a normal polarity of budding (Hicks et al., 1977) in both haploids and diploids (Table 3), and were respiratory competent, as they grew on plates containing glycerol, a nonfermentable carbon source.

Table 2. Effects of MYO4 deletion and overproduction on the frequencies of budded and abnormal cells‡

<table>
<thead>
<tr>
<th>Cell phenotype†</th>
<th>+/+</th>
<th>+/+Δ</th>
<th>ΔΔ</th>
<th>+/+</th>
<th>+/+Δ</th>
<th>ΔΔ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbudded</td>
<td>27</td>
<td>25</td>
<td>24</td>
<td>47</td>
<td>42</td>
<td>37</td>
</tr>
<tr>
<td>Budded</td>
<td>72</td>
<td>75</td>
<td>74</td>
<td>40</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td>8</td>
<td>15</td>
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</tbody>
</table>

‡Wild-type cells (+/+; strain 22AB) and cells that were heterozygous (+/Δ; strain myo4ΔU5) or homozygous (Δ/Δ; strain myo4ΔU5(2A+2D)) for deletion of MYO4 were transformed with a high-copy-number plasmid expressing MYO4 (YEp351-MYO4) or control vector (YEp351).

†Expressed as % of 200 cells for each strain.

Table 3. Effect of MYO4 deletion on budding polarity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Unipolar</th>
<th>Bipolar</th>
<th>Random</th>
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<tbody>
<tr>
<td>Wild-type diploid (+/+†)</td>
<td>54</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>MYO4 deletion homozygote (ΔΔ‡)</td>
<td>54</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>Haploid segregant: +§</td>
<td>99</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Δ</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Δ**</td>
<td>98</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

†22AB.
‡myo4ΔU5(2A+2D).
§myo4ΔU5-2C (MYO4).
¶myo4ΔU5-2B (MYO4).
||myo4ΔU5-2A (myoΔ).
*+myo4ΔU5-2D (myo4A).
Finally, these strains mated and sporulated normally: We found that 40% of the cells sporulated from a cross of MYO4 deletion sister segregants (myo4D U5-2A · myo4D U5-2D), whereas 44% sporulated from a cross of the corresponding wild-type sister segregants (myo4D U5-2B · myo4D U5-2C), and 54% of wild-type diploid control cells (22AB) sporulated (n=600 for all three samples).

We have found by blotting that wild-type cells do indeed express a protein of the predicted molecular mass that labels with anti-Myo4p antibodies (Fig. 7). This protein is overexpressed in cells carrying the gene on a high-copy-number plasmid, but is not present in MYO4 deletion strains. In addition, expression of a truncated MYO4 gene results in the appearance of a smaller protein of the predicted size.

We looked for evidence of functional redundancy or other interaction between Myo4p and the other known yeast myosins by looking for a synthetic (more than additive) phenotype in double mutants. MYO4 deletion strains (myo4D U5-2A or -2D) were crossed with MYO1 deletion strains (BGE1 and myo1D-12B), MYO3 deletion strains (HA1A and HA1C), or a myo2-
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mutant strain (SLY34). In addition, crosses were performed with strains carrying the act1-1 and act1-2 actin mutations (DBY1999 and DBY1993, respectively), with strains carrying mutations in genes whose products are involved in the secretory pathway (all NY strains listed in Table 1) and with strain SLY55 carrying a SMY1 deletion (which is synthetically lethal with myo2-66; Lillie and Brown, 1992). Double mutants were obtained by tetrad dissection. In no case did deletion of MYO4 alter the phenotype of any of these other mutants.

We also looked for evidence of overlapping function by asking whether extra copies of MYO4 could compensate for the defect in the myo2-66 mutant. Introducing MYO4 on either a high-copy-number plasmid (YEp351-MYO4) or low-copy-number plasmid (YCpMYO4) did not overcome the temperature sensitivity of the myo2-66 mutant. (YEp351-MYO4 did cause the phenotypic changes described below for wild-type cells.)

Overexpression of Myo4p (and Myo2p)

As another way of identifying potential function, we have overexpressed Myo4p in yeast, using either wild-type diploid yeast (strain 22AB) or strains heterozygous (strain myo4ΔU5) or homozygous (strain myo4ΔU5(2A+2D)) for deletion of MYO4. Overexpression of Myo4p in all three strains gave

Fig. 6. Micrographs of yeast transformed with MYO4 on a high-copy-number plasmid (YEp351-MYO4), or with control vector (YEp351). (A) Wild-type diploid strain (22AB) + YEp351 (control vector), stained with Calcofluor. Bar, 5 μm, for all micrographs. (B) Diploid MYO4 deletion strain (myo4ΔU5(2A+2D)) + YEp351, stained with Calcofluor. (C) Strain 22AB + YEp351-MYO4, stained with Calcofluor. (D-F) Strain myo4ΔU5(2A+2D) + YEp351-MYO4, stained with (D) Calcofluor or (E,F) DAPI. (G) Haploid wild-type strain (myo4ΔU5-2B) + YEp351-MYO4, stained with Calcofluor. (H) Haploid MYO4 deletion strain (myo4ΔU5-2A) + YEp351-MYO4, stained with Calcofluor.
that 'inactivates' these genes. If the aberrant transformant is omitted, the overexpression phenotype. This is presumably due to some infrequent event will reproducibly show normal plasmid stability, and will not display the overexpression phenotype, we have made a series of truncations and partial deletions (Fig. 3B), and introduced the altered genes on high-copy-number plasmids into the wild-type diploid strain (Fig. 7), and second, there is a high rate of plasmid loss when selection is removed (Table 4, compare control vector to full-length) (Hicks et al., 1977). Some cells were observed to have more than a single nucleus (Fig. 6F), or to lack a nucleus. Calcofluor staining revealed random chitin localization in some cells, and some cells were larger than normal (Fig. 6D). However, a large fraction of the cells in the population were normal-appearing (Table 2, and the single cell population were normal-appearing (Table 2, and the single cell in Fig. 6C). We suspect that these cells may be normal because they have a reduced copy number of the MYO4 plasmid. There are two indications that the plasmid is selected against; first, not much overexpression of Myo4p is seen on western blots, because our antibody lacks the tail domain on western blots, because our antibody.

Table 4. Stability of MYO4-containing plasmids

<table>
<thead>
<tr>
<th>Plasmid†</th>
<th>% Stability*</th>
<th>Plasmid†</th>
<th>% Stability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vector</td>
<td>6±11</td>
<td>MYO1 disruptant§</td>
<td>58±18</td>
</tr>
<tr>
<td>Full-length MYO4</td>
<td>13±19</td>
<td>3±4</td>
<td></td>
</tr>
<tr>
<td>Bgl</td>
<td>30±3</td>
<td>3±4</td>
<td></td>
</tr>
<tr>
<td>Hpa</td>
<td>22±8</td>
<td>3±4</td>
<td></td>
</tr>
<tr>
<td>Xba</td>
<td>66±8</td>
<td>88±4</td>
<td></td>
</tr>
<tr>
<td>EcoRV</td>
<td>72±11</td>
<td>60±7</td>
<td></td>
</tr>
<tr>
<td>N,Nc</td>
<td>0±0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K,Ss</td>
<td>65±18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K,X,X</td>
<td>75±21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Plasmid stability is presented as the average percentage ± s.d. of colonies that scored positive for the plasmid-borne marker out of 25-40 tested for each of 2-8 independent transformants (after ~8 generations in the absence of selection).
†The plasmid designations are the same as used in Fig. 3B.
‡Strain 22AB
§Strain myo1A-12B
¶The large range here is due to one aberrant transformant. An occasional transformant carrying either MYO4 or MYO2 high-copy-number plasmids will reproducibly show normal plasmid stability, and will not display the overexpression phenotype. This is presumably due to some infrequent event that 'inactivates' these genes. If the aberrant transformant is omitted, the mean ± s.d. for the other seven transformants is 6±6.

distinguishable results: an increased frequency of un budded cells and morphological abnormalities (Table 2), including short strings of somewhat elongate cells (Fig. 6C-E). Haploid yeast tended to form clusters rather than strings of cells (Fig. 6G and H); this difference is presumably due to the fact that haploid yeast tend to bud in a unipolar pattern and diploid yeast in a bipolar pattern (Hicks et al., 1977). Some cells were observed to have more than a single nucleus (Fig. 6F), or to lack a nucleus. Calcofluor staining revealed random chitin localization in some cells, and some cells were larger than normal (Fig. 6D). However, a large fraction of the cells in the population were normal-appearing (Table 2, and the single cell population were normal-appearing (Table 2, and the single cell in Fig. 6C). We suspect that these cells may be normal because they have a reduced copy number of the MYO4 plasmid. There are two indications that the plasmid is selected against; first, not much overexpression of Myo4p is seen on western blots, because our antibody lacks the tail domain on western blots, because our antibody.

Table 5. Stability of MYO2- and myo2-66-containing plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>% Stability* at 22°C</th>
<th>% Stability* at 36°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vector†</td>
<td>72±4</td>
<td>71±1</td>
</tr>
<tr>
<td>YEpmyo2</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>YEpmYO2-40</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

*Wild-type strain 22AB was transformed with the indicated plasmids and maintained at either 22°C or 36°C throughout. Plasmid stability is presented as the average percentage ± s.d. of colonies that scored positive for the plasmid-borne marker out of 25 tested for each of two independent transformants (after ~8 generations in the absence of selection).
†YEpl102.

Fig. 7. Western blot of yeast total protein labeled with antibody against Myo4p tail region. (A) Antibody raised against trpE-Myo4p fusion protein and affinity-purified against β-galactosidase-Myo4p fusion protein. (B) Antibody raised against β-galactosidase-Myo4p fusion protein and affinity-purified against trpE-Myo4p fusion protein. Lanes 1, MYO4 deletion strain (myo4ΔU5(2A+2D)) +YEpm351 (control vector); lanes 2, wild-type strain (22AB) + YEpm351; lanes 3, wild type + YEpm351-MYO4; lanes 4, wild type + YEpm351-MYO4(Bgl). Bands of ~170 kDa (lanes 2-4) and ~155 kDa (lane 4) were observed, which corresponded to the calculated molecular masses of full length and truncated Myo4p's, respectively. Although other bands were seen, especially with the antibody used in (A), these were judged to be nonspecific, as they were seen in all four lanes.

To determine what domain of Myo4p causes the overexpression phenotype, we have made a series of truncations and partial deletions (Fig. 3B), and introduced the altered genes on high-copy-number plasmids into the wild-type diploid strain 22AB. We found that truncation of the MYO4 gene at a HpaI site (near the end of the fourth IQ domain in the neck) produced the abnormal phenotype, as did another, less truncated MYO4 (at a BglII site). A more severe truncation (at an XbaI site), encoding a Myo4p missing ~5% of the head domain, did not cause the abnormal phenotype. These results rule out tail function (for example, heterodimerization) as a cause of the effect. Removing most of the head as well as a little of the neck (deleting between NruI and NcoI sites) did not abolish the effect, whereas removing all of the neck as well as a little more head (deleting between KpnI and SspI sites) did abolish the effect. The simplest interpretation of these results is that the neck region may be responsible for the effect. However, we were unable to cause the effect by expressing essentially only the neck region (contained in an XbaI fragment). One plausible explanation is that this latter construct is either not expressed or not stable; we cannot look at the expression of Myo4ps lacking the tail domain on western blots, because our antibody is directed only against this portion of the molecule. The same limitation applies to the most severely truncated Myo4p's; we cannot be certain that they are incapable of conferring an altered phenotype, as we cannot confirm that they are present. For constructs encoding tail regions, we have demonstrated bands of the correct molecular mass on western blots (YEpm351-
MYO4(Bgl) in Fig. 7 and YEp351-MYO4(N,Nc), data not shown).

The results in Table 4 show that the instability of the altered plasmids correlates with their ability to confer an abnormal phenotype. YEp351-MYO4(Hpa), YEp351-MYO4(Bgl), and YEp351-MYO4(N,Nc), which affect the phenotype, are lost at a higher rate than the other altered plasmids, which appear to have no effect on phenotype. Similarly, truncation of MYO2 (resulting in loss of about 1/2 of the tail; see Fig. 3A) did not abolish either its overexpression phenotype or its elevated instability.

We have obtained further evidence that the head domain is not involved in the abnormal phenotype from an examination of the effects of overexpressing a mutated myosin, the myo2-66 allele. We have determined that a single point mutation in the head domain is both necessary and sufficient for the temperature sensitivity of the myo2-66 mutant (S. H. Lillie and S. S. Brown, unpublished data). This mutation lies near an actin-binding face (Rayment et al., 1993), and thus is likely to perturb the ability of Myo2p to bind actin. A high-copy-number plasmid containing the myo2-66 allele (YEpmyo2) was introduced into wild-type cells (strain 22AB) and transformants were maintained at either 22°C or 36°C. (At >30°C, myo2-66 mutants cease normal growth and die.) These transformants displayed the overexpression phenotype not only at 22°C, but also at 36°C, and the YEpmyo2 plasmid showed elevated instability even in cells maintained at 36°C (Table 5). In fact, there was no detectable difference in either the abnormal phenotype or plasmid instability of cells overexpressing wild-type Myo2p or mutated Myo2p at either temperature, indicating that a fully functional head domain is not required.

Because the abnormal phenotype produced by Myo4p or Myo2p overexpression is reminiscent of that produced by disruption or partial deletion of MYO1 (Watts et al., 1987; Rodriguez and Paterson, 1990), we explored the possibility that the overexpression of these myosins is interfering with Myo1p function. However, introduction of YEp352-MYO4 or YEpMYO2 into a MYO1 disruption strain (myo1A-12B) caused the disruption strain to grow more slowly than when transformed with vector alone. As Myo4p or Myo2p overexpression is more deleterious to growth than MYO1 disruption alone, the effects of overexpression cannot be completely accounted for by interference with Myo1p function. Furthermore, YEp352-MYO4 shows the same elevated instability in a MYO1 deletion strain as it does in a wild-type background (Table 4); we would have predicted normal stability in the deletion strain if the only deleterious effect of the plasmid were interference with Myo1p function.

### DISCUSSION

We report the existence of a fourth myosin, Myo4p, in yeast. Myo4p is a class V myosin, based on its sequence similarity to other members of the class, especially in the head domain, and also based on the presence of six IQ domains (putative calmodulin binding sites) in the neck, and a short putative coiled-coil domain in the tail.

We find that deletion of the MYO4 gene produced no detectable phenotypic change. Because it is so similar to Myo2p, another class V myosin in yeast, we initially thought that Myo4p might be redundant with Myo2p. However, Myo4p cannot substitute for Myo2p, even when overexpressed, and MYO4 deletion has no synthetic (more than additive) phenotype with myo2 in double mutants. While myo2 is synthetically lethal with mutations in a subset of genes involved late in the secretory pathway (Govindan et al., 1991), we have found that MYO4 deletion is not synthetically lethal with these mutations, or with mutations in other secretory genes. Thus, redundancy between MYO4 and MYO2 seems unlikely, and perhaps the structural similarity instead reflects some aspect of their function that is similar. For example, Myo2p and Myo4p might be involved in transport of different classes of vesicles or other organelles.

Several lines of evidence indicate that Myo4p is present and active as a myosin in the cell. First, it can be visualized with antibody on western blots. Second, overexpression of Myo4p gives a phenotype similar to that caused either by overexpression of another class V myosin, Myo2p, or by disruption of the MYO1 gene, which encodes a conventional class II myosin. The phenotype in all three cases includes strings or clusters of cells, the increased diameter of some cells, and defects in chitin localization and nuclear segregation.

These similarities in phenotype raised the possibility that overexpressed Myo2p or Myo4p might be competing with Myo1p. However, we know that Myo4p or Myo2p is doing more than simply blocking Myo1p function, since MYO1 disruptants grow more slowly when either of these myosins is overexpressed. Perhaps they are also blocking the function of a second, partly redundant myosin. We can’t judge whether overexpression interferes with any of the other known myosins; defects in Myo3p and Myo4p produce no identifiable phenotypic change, and a defect in Myo2p gives changes (an increase in cell size and chitin delocalization) that partially overlap those produced by defects in Myo1p.

Attempts to immunolocalize Myo4p using the antibodies we’ve described have been unsuccessful to date, despite the use of a number of different fixation and permeabilization procedures and antibody amplification techniques (Pringle et al., 1991; Roberts et al., 1991). We have also attempted to immunolocalize the construct (encoded by YEp352-MYO4(K,Ss)) that lacks head and neck sequences (and can therefore be overexpressed without perturbing morphology) but retains most of the tail (so that it should react with antibody). However, this approach was also unsuccessful.
In summary, studies of a newly identified myosin have revealed that it can function in the cell, but it is not yet clear what its normal function is. Further immunofluorescence (e.g. using an epitope tag) and genetic approaches (e.g. hunts for other genes that are lethal in combination with MYO4) should shed further light on this question.

We thank Chris Davies for originally identifying a fragment of the MYO4 gene and giving it to us, Kathy Corrado for yeast chromosome blots, Jose Rodriguez for the MYO1 disruption strain, Holly Goodson and Jim Spudich for the MYO3 deletion strains, and Trisha Davis for the plasmid-borne calmodulin gene. This work was supported by NIH grant GM46745 and in part by NIH grant no. MO1 RR00042.

REFERENCES


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