

Mlc1p Is a Light Chain for the Unconventional Myosin Myo2p in *Saccharomyces cerevisiae*

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Abstract. In *Saccharomyces cerevisiae*, the unconventional myosin Myo2p is of fundamental importance in polarized growth. We explore the role of the neck region and its associated light chains in regulating Myo2p function. Surprisingly, we find that precise deletion of the six IQ sites in the neck region results in a myosin, Myo2- Δ 6IQp, that can support the growth of a yeast strain at 90% the rate of a wild-type isogenic strain. We exploit this mutant in a characterization of the light chains of Myo2p. First, we demonstrate that the localization of calmodulin to sites of polarized growth largely depends on the IQ sites in the neck of Myo2p. Second, we demonstrate that a previously uncharacterized protein, Mlc1p, is a myosin light chain of Myo2p. *MLC1* (*YGLI06w*) is an essential gene that exhibits

haploinsufficiency. Reduced levels of *MYO2* overcome the haploinsufficiency of *MLC1*. The mutant *MYO2- Δ 6IQ* is able to suppress haploinsufficiency but not deletion of *MLC1*. We used a modified gel overlay assay to demonstrate a direct interaction between Mlc1p and the neck of Myo2p. Overexpression of *MYO2* is toxic, causing a severe decrease in growth rate. When *MYO2* is overexpressed, Myo2p is fourfold less stable than in a wild-type strain. High copies of *MLC1* completely overcome the growth defects and increase the stability of Myo2p. Our results suggest that Mlc1p is responsible for stabilizing this myosin by binding to the neck region.

Key words: myosin • polarized • stability • Myo4 • cytokinesis

THE *Saccharomyces cerevisiae* unconventional myosin Myo2p is a member of the class V myosins and has been implicated in vesicle movement and polarized growth (Johnston et al., 1991; Govindan et al., 1995). Homologues of Myo2p include mouse *dilute*, which plays a role in melanosome transport (Provance et al., 1996; Nascimento et al., 1997), and chicken myosin V, which has a role in neuron filopodial extension (Wang et al., 1996). Myo2p is essential for growth of *S. cerevisiae*, and it localizes to the bud tip during bud formation and to the bud neck during cytokinesis (Brocknerhoff et al., 1994; Lillie and Brown, 1994). A temperature-sensitive mutation in *MYO2* confers defects in polarized growth and in vacuole inheritance but not in general secretion (Johnston et al., 1991; Govindan et al., 1995; Hill et al., 1996).

All myosins have at least one light chain that binds to the myosin heavy chain via a light chain binding motif called an IQ site (Cheney and Mooseker, 1992; Xie et al., 1994). IQ sites are often found in tandem repeats between the head-motor domain and the tail domain (Cheney and

Mooseker, 1992; Rayment et al., 1993). IQ sites are ~25–amino acid residue motifs that bind calmodulin or myosin light chains. In many cases, the binding of these small EF-hand proteins activates the Mg^{2+} ATPase activity of myosins. For example, calmodulin is required for the Mg^{2+} ATPase activity of chicken myosin V (Espindola et al., 1992). Furthermore, light chains perform structural roles by affecting myosin head orientation as well as orientation of light chains to each other. As an example, the light chains of scallop myosin II are required to stabilize Ca^{2+} binding by the myosin head domain and alter the myosin head orientation (Fromherz and Szent-Gyorgyi, 1995). Myo2p has six tandem IQ sites (Johnston et al., 1991).

We have previously shown that calmodulin binds to Myo2p to perform an essential function in polarized growth (Brocknerhoff et al., 1994). Not only is calmodulin present at sites of polarized growth (Brocknerhoff and Davis, 1992), but calmodulin and Myo2p have direct physical contact through the IQ sites in the neck of Myo2p (Brocknerhoff et al., 1994). *CMD1* mutants show allele-specific synthetic lethality with the mutant *myo2-66*, thus suggesting that calmodulin and Myo2p share an essential function.

Interestingly, both class V myosins in *S. cerevisiae*, Myo2p and Myo4p, confer deleterious effects on cell

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Table I. Plasmids Used in This Study

Plasmid	Parent vector	Relevant markers and construction*	Reference or source
pBluescriptII KS (+)		amp ^r fl origin	Stratagene
pGEX-2T		<i>Ptac</i> with gene encoding GST	Pharmacia
pQE30		<i>Ptac</i> with 6XHis polylinker	Qiagen
pRS306		<i>URA3</i> fl origin	(Sikorski and Hieter, 1989)
pRS315		<i>CEN6 ARSH4 LEU2</i> fl origin	(Sikorski and Hieter, 1989)
pRS316		<i>CEN6 ARSH4 URA3</i> fl origin	(Sikorski and Hieter, 1989)
pGF27	pRS304	2- μ m origin (YEp24 fragment) inserted at AatII site	G. Zhu
pGF29	pRS306	2- μ m origin (YEp24 fragment) inserted at AatII site	G. Zhu
pJG28	pSB5	amp ^r trc promoter, <i>CMD1</i>	J. Geiser
pJP10-2B	YCp50	<i>URA3 MYO2 CEN4 ARS1</i>	(Johnston et al., 1991)
pLI831	pBluescriptII SK (+)	<i>ADE3</i>	E. Muller
myo4 Δ ::URA3	pBluescriptII KS (+)	<i>myo4</i> Δ :: <i>URA3</i>	S. Brown
pRS23	pBluescriptII SK (+)	5.6-kb ClaI fragment of <i>MYO2</i>	This study
pRS28	pRS27	<i>myo2</i> Δ :: <i>TRP1</i>	This study
pRS29	pRS25	5.6-kb ClaI fragment of <i>MYO2</i>	This study
pRS31	pGF29	5.6-kb ClaI fragment of <i>MYO2</i>	This study
pRS43	pRS29	NheI site removed	This study
pRS50	pRS37	2- μ m origin (YEp24 fragment) inserted at NotI site	This study
pRS72	pRS43	NcoI site added at codon 1 of <i>MYO2</i>	This study
pRS172	pRS43	Internal deletion of <i>MYO2</i> (S787G Δ aa788–927) also known as <i>MYO2-D6IQ</i>	This study
pRS174	pRS306	5.4-kb ClaI fragment of <i>MYO2-D6IQ</i>	This study
pRS221	pGF29	5.4-kb ClaI fragment of <i>MYO2-D6IQ</i>	This study
pRS276	pBluescriptII KS (+)	3.3-kb BamHI fragment carrying <i>MLC1</i>	This study
pRS286	pRS285	<i>mlc1</i> Δ :: <i>TRP1</i>	This study
pRS289	pRS316	1.4-kb BamHI-SacI fragment of <i>MLC1</i>	This study
pRS290	pGF27	1.4-kb BamHI-SacI fragment of <i>MLC1</i>	This study
pRS296	pQE30	amp ^r promoter 6XHis- <i>MLC1</i>	This study
pRS321	pRS315	1.4-kb BamHI-SacI fragment of <i>MLC1</i>	This study
pSB6	pACYC177	kan ^r lysis genes of lambda	(Brockerhoff et al., 1994)
pSB20	pGEX-3X	<i>MYO2</i> (aa740–1457) fused to GST	(Brockerhoff et al., 1994)
pSB21	pGEX-3X	<i>MYO2</i> (aa740–1116) fused to GST	(Brockerhoff et al., 1994)
pSB24	pGEX-3X	<i>MYO2</i> (aa790–924) fused to GST	(Brockerhoff et al., 1994)
pSB25	pGEX-3X	<i>MYO2</i> (aa740–833) fused to GST	(Brockerhoff et al., 1994)
pSB27	pGEX-3X	<i>MYO2</i> (aa247–740) fused to GST	(Brockerhoff et al., 1994)
pTD28	pTD17	2- μ m origin <i>LYS2 CMD1</i>	(Davis and Thorner, 1989)
pTD29	YEp24	2- μ m origin <i>LYS2</i>	(Geiser et al., 1993)

*Unless otherwise stated, all markers from the parent plasmid are present in the new construct. *MYO2- Δ 6IQ* encodes an internal deletion in Myo2p with the mutation S787G and missing amino acids 787–927. *MLC1* is identical to the *Saccharomyces cerevisiae* open reading frame *YGL106w*.

growth when overexpressed (Haarer et al., 1994). Myo4p localizes to the bud and is essential for the polarized distribution of the asymmetry determinant, Ash1p (Bobola et al., 1996; Jansen et al., 1996). *MYO4* is not essential for growth (Haarer et al., 1994). The cause of growth defects when either *MYO2* or *MYO4* is overexpressed is unknown.

Here we investigate the role of light chains in the function of Myo2p. Localization of calmodulin to sites of cell growth depends on the neck region of Myo2p. The *S. cerevisiae* genome sequencing project revealed a small protein with similarities to calmodulin and myosin light chains. We show that this protein, which we name Mlc1p (mycosin light chain), binds to the myosin neck and regulates the stability of Myo2p.

Materials and Methods

Plasmids

The plasmids used in this study are listed in Table I.

Plasmid pRS28, used to precisely delete *MYO2*, was made in several steps. The 5.6-kb ClaI fragment of pJP10-2B, containing *MYO2*, was

cloned into the ClaI site of pBluescriptII KS⁺ (Stratagene, La Jolla, CA) to make pRS23. In pRS24, the 4.1-kb NdeI-AflIII fragment of *MYO2* was removed. Plasmid pRS24 was digested with EcoRI, the ends were filled in with the Klenow fragment of DNA polymerase to destroy the EcoRI site, and the ends were ligated with T4 DNA ligase to make pRS26. The remaining coding sequence of *MYO2* in pRS26 was replaced with an EcoRI site by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) using the primer MYO2D (5'-CTCGCGCCATCAGTTGAATTCTATACGCTGTAATATCTGCTG-3'), creating plasmid pRS27. In the plasmid pRS28, a 0.8-kb EcoRI fragment containing *TRP1* (Davis et al., 1986) was cloned into the EcoRI site of pRS27.

The plasmid shuffle used in this study required a plasmid encoding *ADE3* and *MYO2*. A 5.6-kb ClaI fragment of *MYO2* was cloned into the SmaI site of pLI831 (Geiser et al., 1993) to make pRS37. The shuttle vector pRS50 contains a 2- μ m origin from a 2.1-kb EcoRI fragment of YEp24 ligated into the NotI site of pRS37. In both plasmids, the 5' overhangs were filled in by treatment with the Klenow fragment of DNA polymerase.

Additional plasmids containing *MYO2* were made carrying the *URA3* selectable marker. Plasmid pRS25 is a derivative of pRS316 (Sikorski and Hieter, 1989) in which the HindIII-NotI fragment of the polylinker was removed, the 5' overhangs were filled in with Klenow, and the plasmid was recircularized with T4 DNA ligase. The plasmid pRS29 contains the 5.6-kb ClaI fragment of *MYO2* from plasmid pJP10-2B cloned into the ClaI site of pRS25. In pRS29, the coding sequence of *MYO2* runs in

the same direction as the fl origin. For later studies, the *NheI* site of pRS29 was removed by digestion with *NheI*, filling in with Klenow, and reclosing with T4 DNA ligase to create pRS43. The high-copy number plasmid pRS31 was created by cloning the 5.6-kb *ClaI* fragment of *MYO2* into the *ClaI* site of pGF29, a 2- μ m plasmid.

Deletion of the region encoding the neck of *MYO2* was made by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Because of the large size of *MYO2*, a cassette was created containing the middle one-third of the gene. The 2.6-kb *BamHI*-*EcoRI* fragment of *MYO2* was cloned into the *BamHI* and *EcoRI* sites of pBluescriptII KS⁺ to make pRS78. The first step towards deleting the six IQ sites was to introduce *NarI* restriction endonuclease cleavage sites into the coding sequence of *MYO2* at either end of the region encoding the IQ sites. The oligonucleotide *NAR1* (5'-GCAATAAGATGCATAATGGCGCCATTGTTATGATCCAGAAG-3') results in the addition of a glycine between amino acid residues 786 and 787 as well as the mutation S787A. The oligonucleotide *NAR4* (5'-GAAATTGAAACAATTGAAGGGCGCCGACGCTAAATCAGTTAATC-3') results in the addition of a glycine between amino acids 927 and 928. After mutating pRS78 using *NAR1* and *NAR4*, the resulting plasmid was digested with *NarI* and reclosed using T4 DNA ligase. This mutant cassette was then ligated to *BamHI*- and *EcoRI*-cut pRS43 to create the plasmid pRS172, which encodes a Myo2p with an S787G mutation and lacks only amino acids 788–927. This mutant was named *MYO2- Δ 6IQ*. Plasmid pRS221 has the 5.4-kb *Asp718*-*SacI* fragment containing *MYO2- Δ 6IQ* cloned into pGF29.

The plasmid pRS174 was created to integrate *MYO2- Δ 6IQ* into the genome of *S. cerevisiae*. pRS174 was made by ligating the 5.4-kb *ClaI* fragment of pRS172 containing *MYO2- Δ 6IQ* into the *ClaI* site of pRS306.

The gene *YGL106w* encodes a 149-amino acid protein with similarities to calmodulin. Based on the work described in this paper, we have renamed *YGL106w* as *MLC1*. The 3.3-kb *BamHI* fragment of lambda PM-3353 (American Type Culture Collection, Rockville, MD) was cloned into the *BamHI* site of pBluescriptII KS⁺, creating plasmid pRS276. Plasmid pRS287 is a derivative of pRS276, in which the 2.0-kb *NdeI*-*NotI* fragment was removed, 5' overhangs were filled in with Klenow, and plasmid was recircularized with T4 DNA ligase. In plasmid pRS287, *MLC1* is the only open reading frame remaining in the pBluescript polylinker.

The plasmid to delete *MLC1* precisely was made in a similar way to the *myo2 Δ* construct. Plasmid pRS284 was made by digesting pRS276 with *EcoRI*, filling in the 5' overhangs with Klenow, and recircularizing the plasmid with T4 DNA ligase. The coding sequence of *MLC1* was replaced with an *EcoRI* site by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) using primer *MLC1D* (5'-CTAAATTTGCAGTTCGCACTCTCAGAATTCTGTTATTCTATGTATGTGCG-3'), creating pRS285. A 0.8-kb *EcoRI* fragment containing *TRP1* (Davis et al., 1986) was cloned into the *EcoRI* site of pRS285 to make pRS286. In pRS286, the *TRP1* gene is in the same orientation as the original *MLC1*.

Plasmid pRS296, designed to express 6XHis-tagged *MLC1* in *Escherichia coli*, was made in several steps. An *NcoI* site was placed at the first codon of *MLC1* by oligonucleotide-directed mutagenesis using primer *MLC1-NCOI* (5'-GGCTCTGGTGGCTGCCATGGTTATTCTATGTATGTGC-3') (Kunkel et al., 1987) to make pRS291. This mutagenesis also created the mutation S2A. The *MLC1* fragment was made by digesting pRS291 with *NcoI*, filling in using the Klenow fragment of DNA polymerase, digesting with *SacI*, and purifying the 0.8-kb fragment. Plasmid pRS296 was then made by digesting pQE30 (Qiagen, Inc., Chatsworth, CA) with *BamHI*, filling in using the Klenow fragment of DNA polymerase, digesting with *SacI*, and ligating in the 0.8-kb *MLC1* fragment.

Media and Strains

Media for growth of *S. cerevisiae* and *E. coli* were described previously (Zhu et al., 1993). The yeast strains used in this study are listed in Table II. Genetic manipulations and transformations were performed essentially as described previously (Sherman et al., 1986). RSY2 carrying the deletion of *MYO2* was created by one-step gene replacement (Rothstein, 1991) using the plasmid pRS28 digested with *BglIII* and *ClaI* transformed into RSY1. Because strains containing *ade3 Δ -100* are histidine auxotrophs, *ADE3* on a plasmid can be selected by histidine prototrophy (Jones and Fink, 1982). RSY2 was transformed with pRS50 carrying *ADE3* and *MYO2*. The strain RSY2-60B has *MYO2* deleted from the genome and carries pRS50.

The mutant *MYO2- Δ 6IQ* was integrated into yeast by two-step gene replacement (Rothstein, 1991) using plasmid pRS174 digested with *BamHI*. Strains RSY21 and RSY22 were shown to contain *MYO2- Δ 6IQ* by PCR and by Southern blot analysis. RSY22 was mated to strain

MMY28 carrying an integrated form of *GFP-CMD1* (Moser et al., 1997) to make the diploid strain RSY33. A *myo4 Δ ::URA3* construct (a gift from Susan Brown, University of Michigan, Ann Arbor, MI) was transformed into RSY33 to create strain RSY38, heterozygous for *GFP-CMD1*, *MYO2- Δ 6IQ*, and *myo4 Δ* .

Strain RSY105 carrying a deletion of *MLC1* was difficult to create because *MLC1* displays haploinsufficiency. To create the disruption, we first transformed JGY46 with plasmid pRS289, carrying the *MLC1* gene. The resulting strain had three functioning copies of *MLC1*. If one copy of *MLC1* were removed by one-step gene replacement, then the resulting strain would still have two copies of the gene. The 1.7-kb *BamHI*-*NdeI* fragment of plasmid pRS286 encoding *mlc1 Δ ::TRP1* was transformed into JGY46(pRS289). Of the 42 colonies obtained, four were unable to lose the *URA3*-marked *MLC1* plasmid pRS289 when streaked onto minimal medium containing 5'-fluoro orotic acid (5'-FOA).¹ These four colonies were the likely candidates for heterozygous *mlc1 Δ* strains. To test this hypothesis, the four strains were transformed with the *LEU2*-marked *MLC1* plasmid pRS321 and streaked onto minimal medium containing 5'-FOA. Three of the four strains were able to lose pRS289 when given an alternative source of *MLC1* from pRS321. RSY105 was shown to be heterozygous *MLC1/mlc1 Δ ::TRP1* by Southern blot analysis. RSY105 must carry a plasmid source of *MLC1* to survive.

To study the null phenotype of *mlc1 Δ* , strains RSY105(pRS289) and JGY46 were sporulated on SpoIII medium, and 50 tetrads were dissected from each strain. After 24 h, spores were examined for germination and colony growth. Images of spores and colonies were obtained as described below using a fluorescent microscope (model AxioPlan; Carl Zeiss, Inc., Thornwood, NY) with 0.3-s, low-light exposures. Germinating spores were transferred to 20 μ l minimal medium containing 3.7% formaldehyde. After 60 min at room temperature, 4,6'-diamidino-2-phenylindole (DAPI) was added to a final concentration of 50 μ g/ml, and nuclei were examined as described below.

Immunoblot Analysis

SDS-PAGE and immunoblot analysis were performed as described (Geiser et al., 1991) with the following exceptions. Strains to be analyzed were grown to 50 Klett units in the appropriate medium. After centrifugation, cells were washed in lysis buffer (50 mM Tris, pH 8.0, 1 \times proteinase inhibitor cocktail [Drubin et al., 1988]). Cells were lysed by mixing equal volumes of cells, glass beads (Sigma Chemical Co., St. Louis, MO), and lysis buffer and vortexing five times for 1 min alternating with 1-min incubations on ice. After centrifugation for 15 min in a microcentrifuge at 4°C, pellets were washed once in lysis buffer and solubilized using cracking buffer (10 mM sodium phosphate, pH 7.2, 1% β -mercaptoethanol, 1% SDS, and 6 M urea). For detection of Myo2p, 30 μ g of protein was loaded into each lane of a 6% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes using a wet-transfer apparatus (Bio-Rad Laboratories, Hercules, CA) following the instructions provided by the manufacturer. The membranes were incubated with a 1:400 dilution of affinity-purified anti-Myo2p antibody (Lillie and Brown, 1994). Secondary antibody was blotting grade goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) at a 1:5,000 dilution. Signal was detected using Renaissance luminol reagent (DuPont/NEN, Boston, MA) and Hyperfilm-MP (Amersham Corp., Arlington Heights, IL).

Gel Overlays

Production of ³⁵S-labeled 6XHis-Mlc1p was as follows. The *E. coli* strain GM1 (Coulondre and Miller, 1977) containing the plasmids pRS296 and pSB6 was grown in 30 ml of M9 medium (Miller, 1972) with 1.0 mM MgCl₂ (instead of MgSO₄), 0.4% glucose, 1 μ g/ml thiamine, 50 μ g/ml ampicillin, 15 μ g/ml kanamycin, and 8 mCi carrier-free Na³⁵SO₄ (DuPont/NEN) to 20 Klett units and then induced by adding isopropyl- β -D-thiogalactopyranoside to a final concentration of 2 mM. After 5 h at 37°C, the cells were collected by centrifugation and resuspended in 400 μ l lysis buffer (50 mM Tris, pH 7.5, 1 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride). The sample was frozen and thawed several times, alternating

1. *Abbreviations used in this paper:* 5'-FOA, 5'-fluoro orotic acid; DAPI, 4',6'-diamidino-2-phenylindole; GFP, green fluorescent protein; GST, glutathione-S-transferase.

Table II. Yeast Strains

Strain	Genotype*	Reference
CRY1	<i>MATa ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	R. Fuller (Stanford University, CA)
JGY46	<i>MATa/MATα ade2-1oc/ade2-1oc can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1</i>	(Geiser et al., 1991)
EMY55-5D	<i>MATa ade2-1oc ade3Δ-100 can1-100 cyh2^r his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1</i>	E. Muller (University of Washington, Seattle, WA)
EMY55-6B	<i>MATα ade2-1oc ade3Δ-100 can1-100 cyh2^S his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1</i>	E. Muller (University of Washington)
MMY28	<i>MATa ade2-1oc ade3Δ-100 can1-100 (S65T-GFP)-CMD1 his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1</i>	(Moser et al., 1997)
RSY1	EMY55-5D X EMY55-6B	This study
RSY2	<i>MATa/MATα ade2-1oc/ade2-1oc ade3Δ-100/ade3Δ-100 can1-100/can1-100 cyh2^r/cyh2^S his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 MYO2/myo2Δ::TRP1 trp1-1/trp1-1 ura3-1/ura3-1</i>	This study
RSY2-60B	<i>MATa ade2-1oc ade3 Δ-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::HIS3 myo2Δ::TRP1 trp1-1 ura3-1</i> carrying plasmid pRS50	This study
RSY21	<i>MATa ade2-1oc can1-100 his3-11,15 leu2-3,112 MYO2-D61Q trp1-1 ura3-1</i>	This study
RSY22	<i>MATα ade2-1oc can1-100 his3-11,15 leu2-3,112 MYO2-D61Q trp1-1 ura3-1</i>	This study
RSY38	<i>MATa/MATα ade2-1oc/ade2-1oc ADE3/ade3Δ-100 can1-100/can1-100 CMD1/GFP-CMD1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 MYO2/MYO2-Δ61Q MYO4/myo4Δ::URA3 trp1-1/trp1-1 ura3-1/ura3-1</i>	This study
RSY38-9D	<i>MATa ade2-1oc ade3Δ-100 can1-100 GFP-CMD1 his3-11,15 leu2-3,112 lys2Δ::HIS3 MYO2-Δ61Q trp1-1 ura3-1</i>	This study
RSY38-16A	<i>MATa ade2-1oc ade3Δ-100 can1-100 GFP-CMD1 his3-11,15 leu2-3,112 lys2Δ::HIS3 myo4Δ::URA3 trp1-1 ura3-1</i>	This study
RSY38-16C	<i>MATα ade2-1oc ade3Δ-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::HIS3 MYO2-Δ61Q myo4Δ::URA3 trp1-1 ura3-1</i>	This study
RSY38-17C	<i>MATα ade2-1oc ade3Δ-100 can1-100 GFP-CMD1 his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1</i>	This study
RSY105	<i>MATa/MATα ade2-1 oclade2-1 oc can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 MLC1/mcl1D::TRP1 trp1-1/trp1-1 ura3-1/ura3-1</i> carrying plasmid pRS289	This study
RSY105-6A	<i>MATα ade2-1oc can1-100 his3-11,15 leu2-3,112 mcl1Δ::TRP1 trp1-1 ura3-1</i> carrying plasmid pRS289	This study
RSY107	RSY21 X RSY105-6A carrying plasmid pRS289	This study
RSY112	<i>MATa/MATα ade2-1oc/ade2-1oc ade3Δ-100/ade3Δ-100 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 MLC1/mcl1Δ::TRP1 MYO2/myo2Δ::TRP1 trp1-1/trp1-1 ura3-1/ura3-1</i> carrying plasmid pRS289	This study

* *GFP-CMD1* encodes a fusion protein of green fluorescent protein and calmodulin.

between -20 and 20°C to lyse the cells, and then centrifuged at $10,000\text{ g}$ for 30 min in a 4°C microcentrifuge. The supernatant fraction was batch mixed for 10 min with $700\ \mu\text{l}$ preequilibrated Ni-NTA beads (Qiagen, Inc.). The beads were poured to form a column and washed with 10 ml lysis buffer containing 25 mM imidazole. Purified 6XHis-Mlc1p was eluted from the column using lysis buffer containing 500 mM imidazole. The imidazole buffer was exchanged with 100 mM ammonium bicarbonate with a PD-10 G25 column (Pharmacia Biotech, Piscataway, NJ). Protein was stored at -80°C until needed. Protein concentration was determined using bicinchoninic acid (Sigma Chemical Co.). The purified ^{35}S -labeled 6XHis-Mlc1p had a specific activity of 27 dpm/fmol .

Purified ^{35}S -labeled calmodulin was made as previously described (Brocknerhoff et al., 1992), except that only $8\text{ mCi Na}^{35}\text{SO}_4$ was used in the labeling. The purified ^{35}S -labeled calmodulin had a specific activity of 39 dpm/fmol .

The gel overlay assays for calmodulin and Mlc1p binding were performed as previously described (Brocknerhoff et al., 1994) with slight modifications. In brief, extracts were made from the *E. coli* strain GM1 (Coulondre and Miller, 1977) containing either plasmid pGEX-2T, pSB20, pSB21, pSB24, pSB25, or pSB27. Approximately $30\ \mu\text{g}$ total protein was loaded into each lane for SDS-PAGE. Proteins were transferred to Immobilon membranes (Millipore Corp., Bedford, MA) using a wet-transfer apparatus (Bio-Rad Laboratories) following the instructions provided by the manufacturer. After transfer, the proteins were renatured by washing the Immobilon membranes for a total of 40 min (four changes) in buffer A (20 mM Hepes, $\text{pH } 7.2$, 100 mM NaCl) and blocked for 6 h in buffer A containing 3% bovine serum albumin and 0.05% Tween-20. The mem-

branes were probed for 16 h at 21°C with either 140 mM ^{35}S -labeled 6XHis-Mlc1p or 156 mM ^{35}S -labeled calmodulin in buffer C (buffer A with 5 mM CaCl_2 and 0.05% Tween-20). The membranes were then washed for 40 min (four changes) in buffer C. Next, the membranes were air dried, dipped in 7% 2,5-diphenyloxazole in acetone, air dried, and exposed to Hyperfilm-MP (Amersham Corp.) for $5-8\text{ d}$.

Northern Analysis of MYO2 Transcript

Total mRNA was purified from strains of CRY1 carrying either pRS43, pRS172, pRS31, or pRS221 using a previously described procedure (Wise, 1991). From 500-ml cultures, $\sim 7.0\text{ mg}$ RNA was obtained. Total mRNA was purified from the samples using the Promega PolyA Tract mRNA Isolation System I (Madison, WI) with MagneSphere particles. Approximately $50\ \mu\text{g}$ poly A-containing mRNA was purified from each culture.

Northern blot analysis was performed using standard procedures (Sambrook et al., 1989) with the following modifications. $10\ \mu\text{g}$ mRNA was loaded in each lane of a prerun formaldehyde gel. After removing the formaldehyde by washing $2\times$ in DEPC dH_2O , mRNA was transferred to a nitrocellulose membrane using capillary transfer (Sambrook et al., 1989). The membrane was baked at 65°C for 1 h . Probes were made using the 3.4-kb *NcoI*-*EcoRI* fragment of *MYO2* from pRS72 and a PCR product containing a 0.5-kb fragment of *TRX2*. After incubating the membrane with $\sim 1 \times 10^7\text{ cpm}$ of each probe (pooled) for 48 h , the membrane was washed and analyzed using a Molecular Dynamics PhosphorImager (model 400S; Sunnyvale, CA) using 176-micron pixel size.

Indirect Immunofluorescence and Green Fluorescent Protein Fusions

The immunolocalization of Myo2p in strains CRY1 and RSY21 was performed as previously described (Brockerhoff et al., 1994; Lillie and Brown, 1994).

The localization of GFP-Cmd1p in strains RSY38-9D, RSY38-16A, RSY38-16C, and RSY38-17C was performed using a fluorescent microscope (model Axioplan; Carl Zeiss, Inc.) (Moser et al., 1997). 200 cells from each strain were scored for the presence of polarized calmodulin. Calmodulin was judged to be polarized if the fluorescent signal was stronger in the bud than in the mother portion of each cell. Images were captured using an Imagepoint-cooled CCD video camera (Photometrics, Tucson, AZ) fitted to the microscope in conjunction with IP Lab software (Signal Analytics, Vienna, VA). Images for GFP-calmodulin are 5-s exposures. Previous studies have shown that expression of GFP alone in yeast resulted in a uniform distribution of fluorescence throughout the cell, excluding the vacuole (Niedenthal et al., 1996).

Results

Myo2p Functions without a Neck Domain

The neck of Myo2p contains six IQ sites in a tandem array spanning amino acids 790–921 (Johnston et al., 1991). Myo2p is essential for growth, but the six IQ sites are not. The mutant *MYO2-Δ6IQ*, lacking the sequence encoding amino acids 787–927, supports nearly normal growth as the only source of Myo2p, causing only a 10% reduction in growth rate (Fig. 1). An immunoblot confirmed that neck-

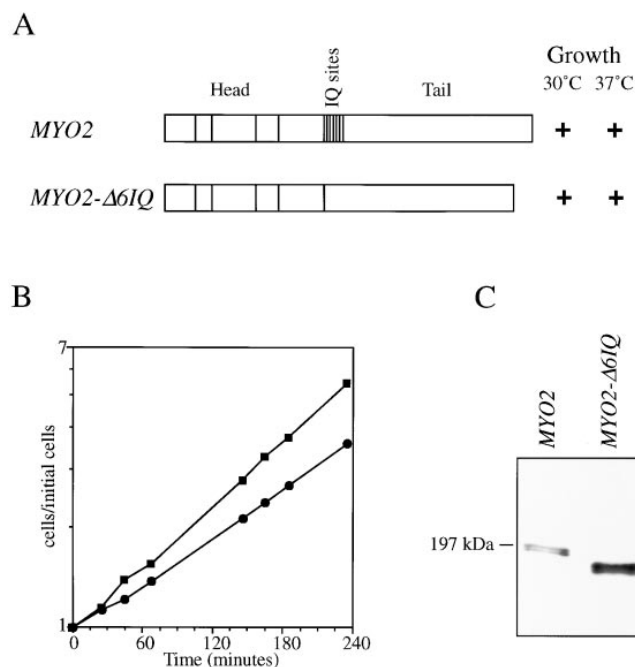


Figure 1. The mutant *MYO2-Δ6IQ* allows growth. Strains carrying wild-type *MYO2* (CRY1) or *MYO2-Δ6IQ* (RSY21) were plated on YPD medium and incubated for 3 d at 30 and 37°C (A). Log-phase cultures were diluted to 10,000 cells/μl in YPD medium, and growth was monitored over 4 h. Cell number was determined on a Coulter counter (Coulter Corporation, Hialeah, FL). (B) ■, wild-type *MYO2* strain, CRY1; ●, *MYO2-Δ6IQ* strain, RSY21. An immunoblot of extracts made from log-phase cultures of strains CRY1 and RSY21 (C). The antibody was anti-Myo2p (Lillie and Brown, 1994).

less Myo2-Δ6IQp is stably produced and that no wild-type Myo2p is present in the strain (Fig. 1 C). The mutant cells have a normal budding index but on average are 10% larger in size. The distribution of bud sizes was similar to that seen in wild-type cultures. The mutant cultures contained about 5% large unbudded cells not found in the wild-type cultures. A strain carrying *MYO2-Δ6IQ* does not grow at 38.5°C, whereas an isogenic wild-type strain grows poorly. A strain carrying *MYO2-Δ6IQ* grows well on medium with or without 1 M sorbitol (data not shown).

The neckless mutant protein localizes indistinguishably from that of wild-type Myo2p (Fig. 2). In both cases, unbudded cells have either no localization or a small patch of Myo2p. In small- and medium-budded cells, the Myo2p is present in a concentrated region at the bud tip. In large-budded cells, the Myo2p is either at the bud tip or at the bud neck (Fig. 2). These results are consistent with previous analysis of Myo2p localization (Brockerhoff et al., 1994; Lillie and Brown, 1994).

Neckless *MYO2-Δ6IQ* Affects Calmodulin Localization

Our previous results strongly suggest calmodulin is a light chain of Myo2p. Calmodulin localizes to the sites of cell

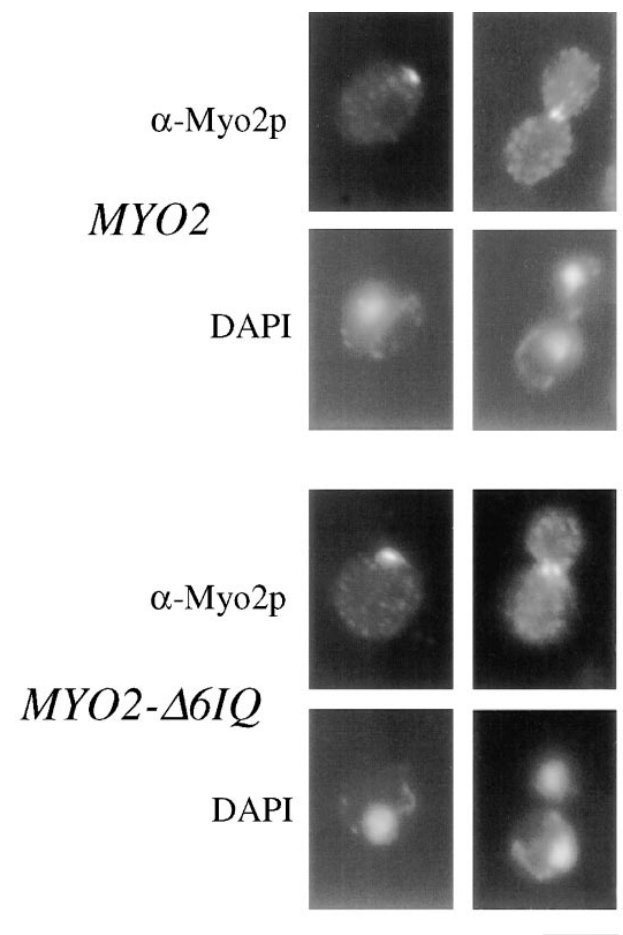


Figure 2. Comparison of the localization of Myo2p and Myo2-Δ6IQp. Cells were stained with affinity-purified anti-Myo2p antibody as described in Materials and Methods. Cells were simultaneously stained with DAPI to stain DNA. Bar, 5 μm.

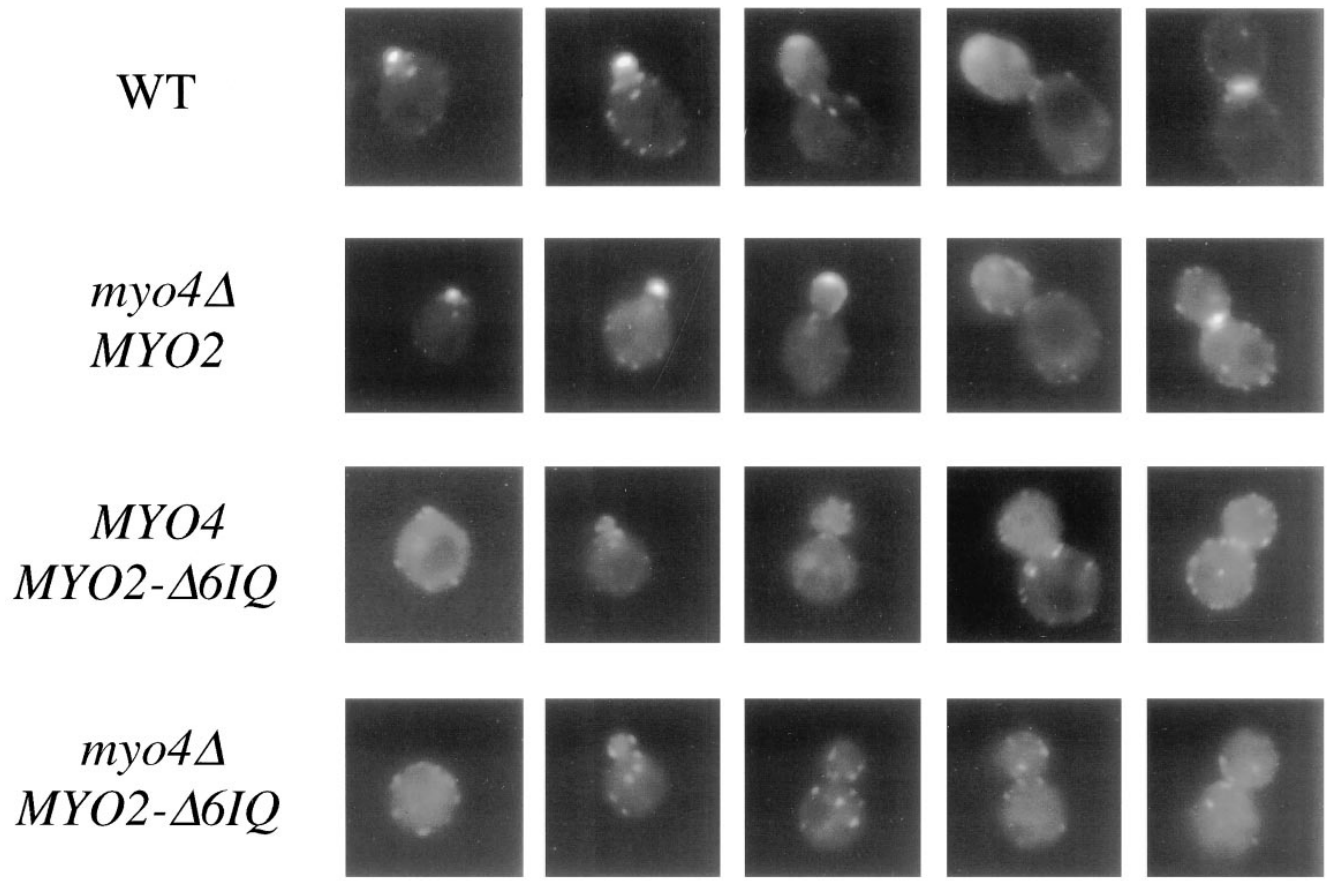


Figure 3. Localization of GFP-calmodulin in *myo4Δ* strains. Cultures of RSY38-17c (WT), RSY38-16A (*myo4Δ MYO2*), RSY38-9D (*MYO4 MYO2-Δ6IQ*), and RSY38-16C (*myo4Δ MYO2-Δ6IQ*) were grown to log phase. Samples were collected, mixed with equal volumes of 1% agarose dissolved in SDC, and mounted on microscope slides for image collection as described in the Materials and Methods. Bar, 5 μ m.

growth, and calmodulin mutants display defects in polarized growth (Brockerhoff and Davis, 1992; Davis, 1992; Ohya and Botstein, 1994). Mutations in *CMD1* exacerbate defects caused by *myo2-66* in an allele-specific manner. The viability of the *MYO2-Δ6IQ* strain allows us to test if the localization of calmodulin at sites of cell growth depends on the six IQ sites of Myo2p. Localization of a fusion of GFP to calmodulin was examined in wild-type and neckless mutant strains. GFP-calmodulin functionally replaces calmodulin and localizes properly (Moser et al., 1997). In a strain carrying wild-type *MYO2*, GFP-calmodulin is found at sites of cell growth in 84% of the cells (Fig. 3 and Table III). This number is equivalent to values obtained by immunolocalization (Brockerhoff and Davis, 1992). The cells without polarized calmodulin are either large-budded cells in the process of moving calmodulin from the bud tip to the bud neck or unbudded cells that have not yet started bud formation. In a strain carrying *MYO2-Δ6IQ*, only 15% of the cells have polarized calmodulin with greatly reduced and more diffuse signal even in these cells. Myo4p localizes diffusely in the yeast bud (Jansen et al., 1996). We tested whether the remaining GFP-calmodulin in the bud of the *MYO2-Δ6IQ* strain was dependent on Myo4p, which is not essential for growth. Strains deleted for *MYO4* and containing wild-type

MYO2 show the same localization of GFP-calmodulin as a wild-type strain containing both *MYO4* and *MYO2*. However, in a strain carrying *MYO2-Δ6IQ* and *myo4Δ*, GFP-calmodulin is polarized in only 4% of the cells (Fig. 3 and Table III). Thus, calmodulin localization to sites of polarized growth is largely dependent on the six IQ sites of Myo2p, although Myo4p contributes to a small extent.

MLC1, a New Light Chain

The newly completed genome sequence for *S. cerevisiae* identified a potential myosin light chain. *YGL106w* encodes a 149-amino acid polypeptide with significant homology to calmodulin and myosin light chains. Based on the results described below, we have renamed this gene *MLC1*. An alignment with vertebrate calmodulin and the most similar light chain reveals homology throughout the

Table III. Calmodulin Localization in Myosin Mutants

Genotype	Polarized calmodulin (%)
Wild type	84
<i>myo2-Δ6IQ</i>	15
<i>myo4Δ</i>	80
<i>myo4Δ myo2-Δ6IQ</i>	4

CaM (H. s) . . . EEQIAEFKFAFSLFDKDGCGTITTKELGTVMRSLGONPHEAELQDMINE V
 MICLC (A. c.) . DEMVDEIKQAFKAEKDKDKGRVNALELGGKIMREMGHNPTTYGELETMTK
 Mlc1p (S. c.) MSATRANKDIFTLFDKKGQCATAKDSSLG DYLRATGYNPTINOLVQDITINADSSLR
 MLE1 (G. g) . . . KEQQDDFKFAFLFLFDRTGDAKITLSQVGDIVRALGONPTNAEINKILGNPSKEE

DADNGTIDFPEFLTMMARKMKT . . . DSEEEIREFARFVFDKDGNGYISAAELR
 ENGGGGVMDVNGFEGMVAKNYSSV . . . DNERDLIEAFQVFDKDGKGFISVMELR
 DASSL . TLDQITGLIEVNEKELDATTKAKTEDFVKAFQVFDKKESTGKVSVDLR
 MNAKKITFEFLPMLQAAANNKD . . . QGTFEDFVEGLRVFDKEGNGTVMGAELR

HVMTNLGEKLTDEEVDDEMIREADIDGCGVNYEEFVQMMTAK
 SILCNMGEKLTDOEVEMLRNACIQDGNVDYTAQVRHMTARDQ
 YMLTGLGEKLTDAEVDDELKGVVEVDSNGEEDYKKEI EDVTRQ
 HVLATLGEKMTVEEVEELMKGQE . DSNGCINYEAFVKHIMSV

Figure 4. Alignment of the predicted amino acid sequence of *MLC1* with three other small EF-hand proteins. Vertebrate calmodulin (*Homo sapiens*) amino acids 7–149, a chicken light chain (*Gallus gallus*, MLE1) amino acids 45–190, and *Acanthamoeba* myosin IC light chain (*A. castellanii*, MICLC) amino acids 7–149 are compared with the complete amino acid sequence of Mlc1p. Amino acid residues sharing identity between Mlc1p and at least one of the other sequences are shaded. Amino acids within potential Ca²⁺-binding loops are labeled with a solid line.

sequences (Fig. 4). Mlc1p shares 35% identity with vertebrate calmodulin, second to only yeast calmodulin among yeast proteins (Davis and Thorner, 1986). Mlc1p is 35% identical to a chicken light chain (MLE1) (Matsuda et al., 1981). Mlc1p is 31% identical to *Acanthamoeba* myosin IC light chain, another unconventional myosin light chain (Wang et al., 1997).

MLC1 Is Essential and Displays Haploinsufficiency

Initial attempts to make a diploid hemizygous for *MLC1* were unsuccessful. Southern blot analysis revealed that one-step gene replacement (Rothstein, 1991) resulted in strains containing at least two wild-type copies of *MLC1* for every one copy of the deletion construct. One possible explanation for this effect is that one copy of *MLC1* per diploid genome is insufficient for viability. This phenomenon is called haploinsufficiency (Wilkie, 1994). We tested if *MLC1* is haploinsufficient by repeating the gene disruption in a strain carrying two copies of *MLC1* in the genome plus one copy of *MLC1* on a plasmid. In this case, we were able to delete one copy of *MLC1* from the genome as described in the Materials and Methods. The hemizygous diploid requires a plasmid carrying *MLC1* for viability (Fig. 5).

Haploid spores that contain the *mlc1Δ* gene disruption require *MLC1* on a plasmid (data not shown). Therefore, *MLC1* is haploinsufficient and essential.

To begin characterizing the phenotype of *mlc1Δ* cells, we examined spores from a hemizygous diploid strain. The diploid requires a plasmid copy of *MLC1* to survive. Any spores receiving the plasmid would grow into colonies even if they carried the genomic copy of *mlc1Δ*. The *mlc1Δ* spores that did not inherit the plasmid were identified as the 20% of the spores that germinated but did not form colonies. The *mlc1Δ* cells grew elongated buds (Fig. 6). Cytokinesis was not complete because removal of the cell wall by treatment with zymolyase did not result in separation of the buds from the mother cells. DAPI staining revealed that the elongated cells have multiple nuclei (Fig. 6 C). Less than 1% of the spores from the control strain formed elongated cells.

Mlc1p Interacts with the Neck of *Myo2p*

Two lines of evidence suggest Mlc1p is a light chain of Myo2p. First, the haploinsufficiency exhibited by *MLC1* is suppressed by reduced copies of *MYO2* (Fig. 5). A diploid strain hemizygous for both *MYO2* and *MLC1* is viable.

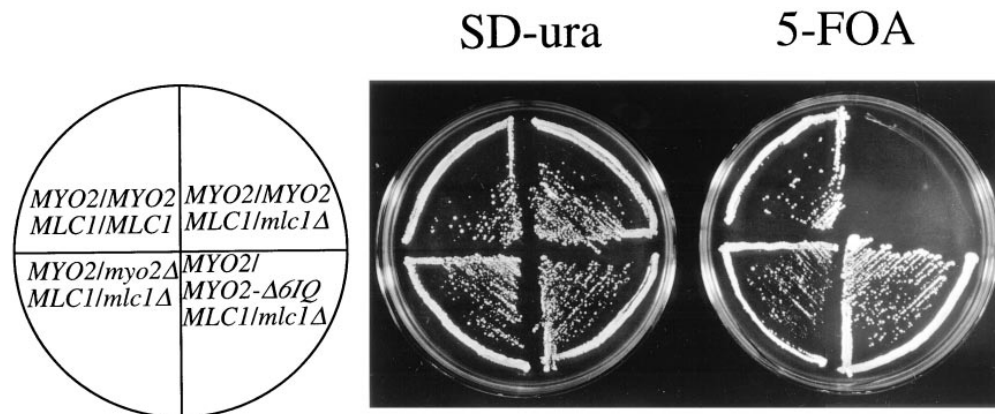


Figure 5. *MLC1* displays haploinsufficiency. Strains JGY46, RSY105, RSY107, and RSY112, each carrying the *MLC1* plasmid pRS289, were streaked onto both minimal medium lacking uracil and minimal medium containing 5'-FOA (which will kill any cells that require the plasmid pRS289). The plates were incubated at 30°C for 3 d.

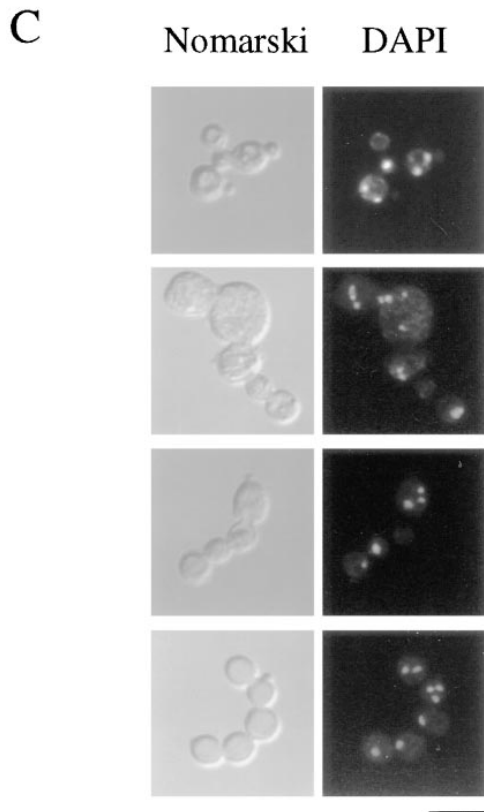
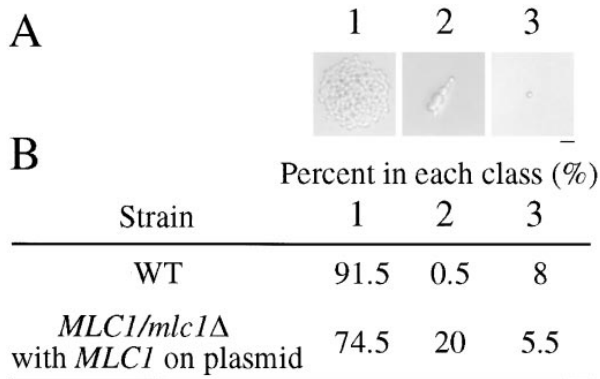


Figure 6. *mlc1Δ* spores have defects in cell separation. Strains JGY46 and RSY105(pRS289) were sporulated and tetrads were dissected. After 24 h, spores were characterized into classes: (1) germinated and formed colonies, (2) germinated and did not form colonies, or (3) did not germinate. Examples of each class are shown (A). 200 spores from each strain were classified (B). Germinated spores from RSY105(pRS289) were stained with DAPI. Elongated spores with DAPI staining are shown (C). Bars, 10 μ m.

Suppression of the haploinsufficiency by hemizygous *MYO2* demonstrates that Mlc1p must ordinarily be present at the minimum level necessary for cell growth. *MYO2-Δ6IQ* can also suppress the haploinsufficiency of *MLC1* (Fig. 5). However, *MYO2-Δ6IQ* cannot bypass the requirement for Mlc1p in a haploid strain (data not shown), so there must be at least one other essential function of Mlc1p in yeast.

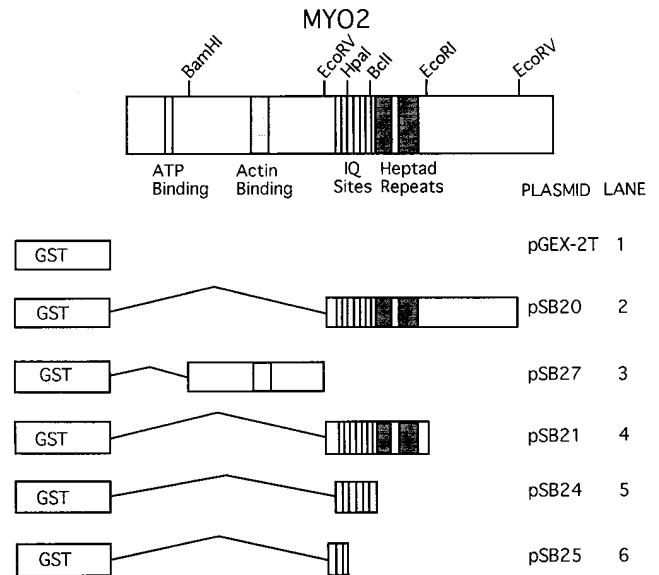


Figure 7. Glutathione-S-transferase (GST)–Myo2p fusion proteins.

Second, Mlc1p binds directly to the IQ sites of Myo2p as assayed by a gel overlay protocol. The constructs used in this experiment are presented in Fig. 7. 6XHis-Mlc1p binds specifically to the fusion proteins that contain IQ sites (Fig. 8 B). As a control, we show calmodulin also binds specifically to fusion proteins that contain IQ sites (Fig. 8 C). Both calmodulin and 6XHis-Mlc1p bind equally well in the presence of Ca^{2+} or EGTA (data not shown). These data demonstrate that Mlc1p has a direct interaction with the IQ sites of Myo2p.

The Toxicity of Overexpressed MYO2 Is Suppressed by MLC1 but Not CMD1

We looked for a functional interaction between Mlc1p and Myo2p. A yeast strain carrying a high-copy number plasmid of *MYO2* grows 40% slower than strains with normal levels of *MYO2* (data not shown). Microscopy has revealed that 5% of cells in the *MYO2* overexpression strain have abnormal bud necks or multiple buds (data not shown). A similar defect in high-copy number *MYO4* strains may be caused by the neck region of Myo4p, and overexpression of calmodulin does not suppress this effect (Haarer et al., 1994). One possibility is that high copies of myosin light chains will overcome defects associated with *MYO2* overexpression.

A strain overexpressing *MYO2* shows a noticeable reduction in growth rate (Fig. 9 A). The same strain overexpressing *MYO2-Δ6IQ* grows normally. Northern blot analysis reveals that both *MYO2* and *MYO2-Δ6IQ* are transcribed to similar steady-state levels in these two strains and at least fivefold over normal levels (Fig. 9 B). Surprisingly, the immunoblot for a strain overexpressing *MYO2* does not show higher levels of Myo2p (Fig. 9, C and D). In fact, Myo2p seems to actually decrease in the strain overexpressing *MYO2*. Overexpressing *MYO2-Δ6IQ* results in high levels of Myo2-Δ6IQp (Fig. 9, C and D).

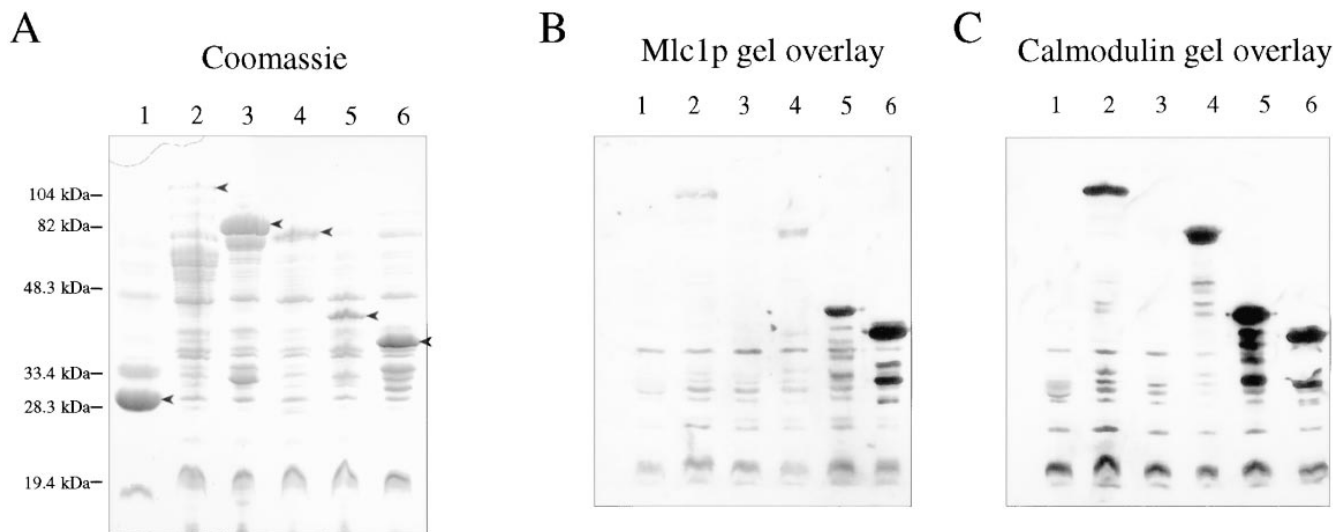


Figure 8. Gel-overlay assays show *in vitro* binding of Mlc1p and calmodulin to the IQ sites of Myo2p. Myo2p fusion proteins were expressed and subjected to SDS-PAGE as described in Materials and Methods. Polyacrylamide gel stained with Coomassie blue. The full-length fusion protein in each extract is marked by an arrow (*A*). Autoradiographs of membranes incubated with ^{35}S -labeled 6XHis-Mlc1p (*B*) and ^{35}S -labeled calmodulin (*C*). The gel overlay assays were performed as described in Materials and Methods. Lanes 1–6 contain extracts of IPTG-induced *E. coli* strain GM1 containing plasmids pGEX-2T, pSB20, pSB27, pSB21, pSB24, and pSB25, respectively. See Fig. 7 and Table I for a description of the fusion proteins.

We hypothesized that high levels of calmodulin or Mlc1p would counteract the toxicity of overexpressed *MYO2*. Overexpressing *CMD1* is unable to overcome the decreased growth rate (Fig. 9 *A*) or the low levels of Myo2p (Fig. 9 *D*) caused by overexpressed *MYO2*. In contrast, a high-copy number plasmid carrying *MLC1* is able to fully suppress the overexpression *MYO2* defects and results in a strain that has high levels of Myo2p (Fig. 9, *A* and *C*). These results indicate that the neck region of *MYO2* is indeed responsible for an overexpression growth defect and that this toxicity is overcome by high levels of Mlc1p.

Mlc1p Stabilizes Myo2p

Because high copies of *MLC1* are able to overcome defects caused by overexpression of *MYO2*, we tested the hypothesis that Mlc1p stabilizes Myo2p. Yeast cultures were analyzed for the stability of Myo2p by following Myo2p breakdown by immunoblot analysis (Fig. 10). In a wild-type strain, Myo2p has a half-life of greater than 8 h. In a strain carrying *MYO2-Δ6IQ*, the Myo2-Δ6IQp has a half-life of greater than 8 h. The neckless Myo2-Δ6IQp is at least as stable as wild-type Myo2p. When overexpressed, the half-life of Myo2p is only 2–3 h. These data explain why the Myo2p levels in the overexpression strain are not increased. In a strain overexpressing *MYO2* and carrying high copies of *MLC1*, the half-life of Myo2p is 4 h. High copies of *MLC1* are able to stabilize Myo2p, thus suggesting Mlc1p is a light chain responsible for stabilizing the neck region of Myo2p.

Discussion

Electron micrographs of the chicken myosin V molecule show two heads connected to a 30-nm stalk. At the proxi-

mal end of each head is a 20-nm neck that likely corresponds to the six IQ sites (Cheney et al., 1993). This region is predicted to be an α -helix as is the neck of class II myosins. In class II myosins, the neck region is stabilized by the binding of the myosin light chains (Rayment et al., 1993; Houdusse and Cohen, 1996). Calmodulin is the previously identified light chain for both the chicken and the yeast myosin V (Cheney et al., 1993; Brockerhoff et al., 1994). However, calmodulin does not stabilize the yeast myosin. Here, we present evidence that *MLC1* (*YGL106w*) encodes a new light chain for the yeast class V myosin Myo2p. Our results argue that Mlc1p binds to the neck region and stabilizes Myo2p.

When overexpressed, Myo2p has a decreased half-life and is toxic to yeast cells. Overproduction of calmodulin does not affect the stability of Myo2p, but overproduction of Mlc1p stabilizes Myo2p and ameliorates the toxicity. The requirement for Mlc1p to stabilize Myo2p is bypassed by deleting the six IQ sites in Myo2p. Thus, the presence of the IQ sites destabilizes Myo2p unless sufficient Mlc1p is present.

Removal of the IQ sites in Myo2p causes a slightly slower growth rate and slightly larger cells. A mutant *Dictpostelium* myosin II heavy chain ΔBLCBS , which lacks the two IQ sites, maintains 20% wild-type motor activity when measured in the sliding filament assay (Uyeda et al., 1996). The ΔBLCBS myosin allows cell division *in vivo* but demonstrates slightly slowed furrow formation during cytokinesis (Zang et al., 1997). Thus, both Myo2-Δ6IQp and the ΔBLCBS myosin confer relatively minor defects. These results indicate that the full efficiency of the myosin is not essential for myosin function *in vivo*.

MLC1 is essential and haploinsufficient. Haploinsufficiency is rarely observed in yeast genes. There are several reasons reduction in the number of gene copies may have

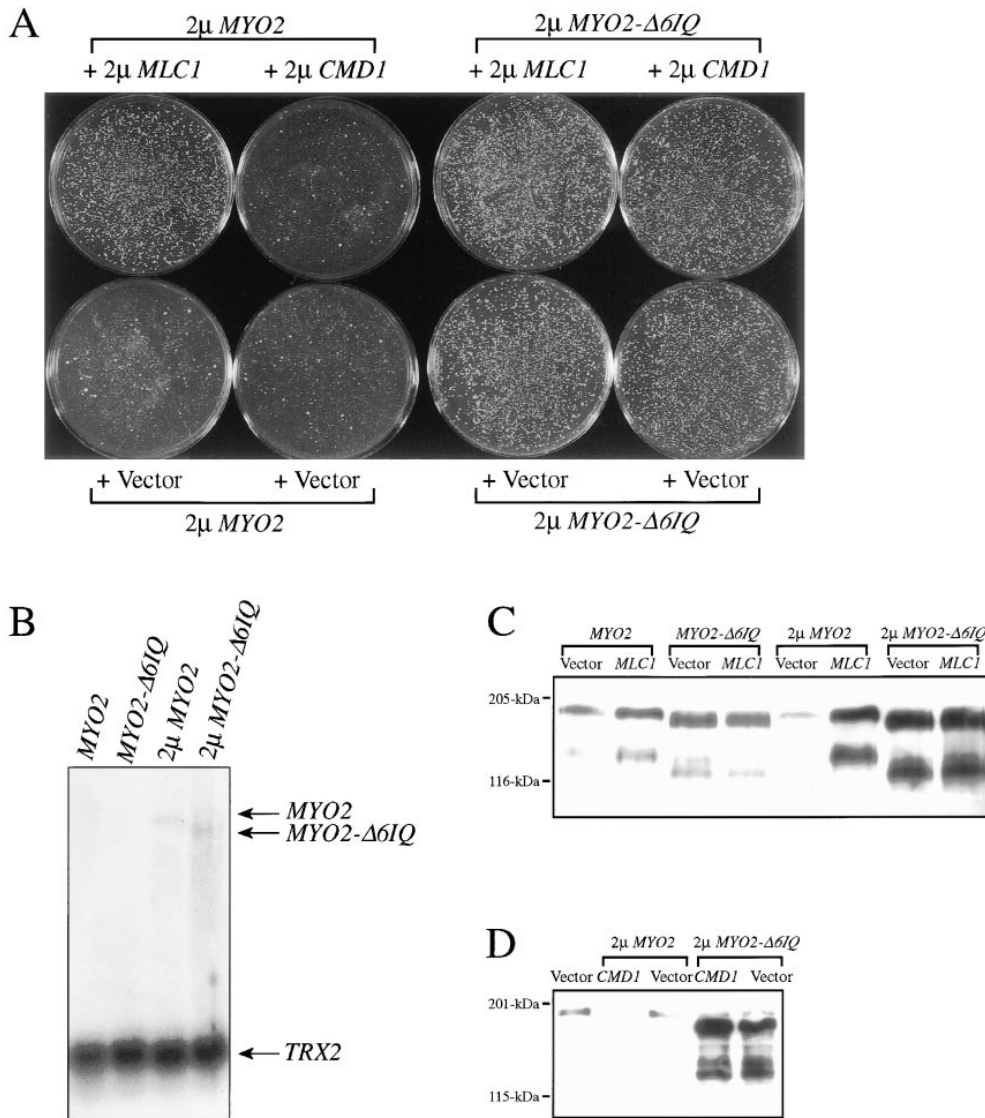


Figure 9. High copies of *MLC1*, but not high copies of *CMD1*, overcome the toxic effects of overexpressed *MYO2*. Plasmids used in these experiments are listed in Table I. *EMY55-5D* was transformed with plasmids encoding high copies of *MYO2*, *MYO2- Δ 61Q*, *CMD1*, or *MLC1*. Plates were incubated for 3 d at 30°C (A). *CRY1* was transformed with pRS43, pRS172, pRS31, or pRS221, and mRNA was collected from log-phase cultures. 10 μ g of polyA-containing RNA samples were analyzed as described in Materials and Methods. Blots were simultaneously hybridized with *MYO2* and *TRX2* ³²P-labeled probes (B). *CRY1* was transformed with the indicated constructs to study the effects of high copies of *MLC1* (pRS290) on strains with one extra copy of *MYO2* (pRS43) or one extra copy of *MYO2- Δ 61Q* (pRS172), or overexpressing *MYO2* (pRS31) or *MYO2- Δ 61Q* (pRS221) (C). *CRY1* was transformed with the indicated constructs to study the effects of high copies of *CMD1* (pTD28) on strains overexpressing *MYO2* (pRS31) or *MYO2- Δ 61Q* (pRS221) (D). Log-phase extracts were made as described in Materials and Methods. Samples were assayed by immunoblot analysis using anti-Myo2p antibody (Lillie and Brown, 1994) to detect steady-state levels of Myo2p.

deleterious effects. In some cases, reduced gene copy number affects regulatory genes working at a threshold level (Wilkie, 1994). An example of this form of regulation is dosage-dependent sex determination in *Drosophila*. Alternately, some proteins may be produced at the minimum level to give proper function. The *ACT1* gene displays temperature-sensitive growth defects and increased osmosensitivity when present at low levels (Shortle et al., 1984). Finally, the stoichiometry of various protein components may be important. Several lines of evidence suggest that a fixed ratio between Mlc1p and Myo2p is required to confer normal growth. First, a diploid yeast strain carrying one copy of *MLC1* can only grow if there is no more than one copy of wild-type *MYO2*. Second, the growth defects associated with a yeast strain overexpressing *MYO2* are

eliminated by adding extra copies of *MLC1*. Finally, removal of the IQ binding sites from Myo2p rescues the haploinsufficiency exhibited by *MLC1*.

Myo2- Δ 61Qp does not overcome a requirement of Mlc1p for cell viability. This observation does not address the essential nature of the interaction between Mlc1p and Myo2p. It merely demonstrates that Mlc1p must have at least one essential function that does not involve binding to the neck of Myo2p. None of the other four myosins in yeast are essential for growth (Brown, 1997), suggesting that Mlc1p has an essential function unrelated to myosins. In viable *mlc1 Δ* cells show a striking defect in cytokinesis, resulting in enlarged, multinucleate cells. This phenotype is identical to defects detected in cells deleted for *IQG1*, which encodes a newly discovered IQGAP family member

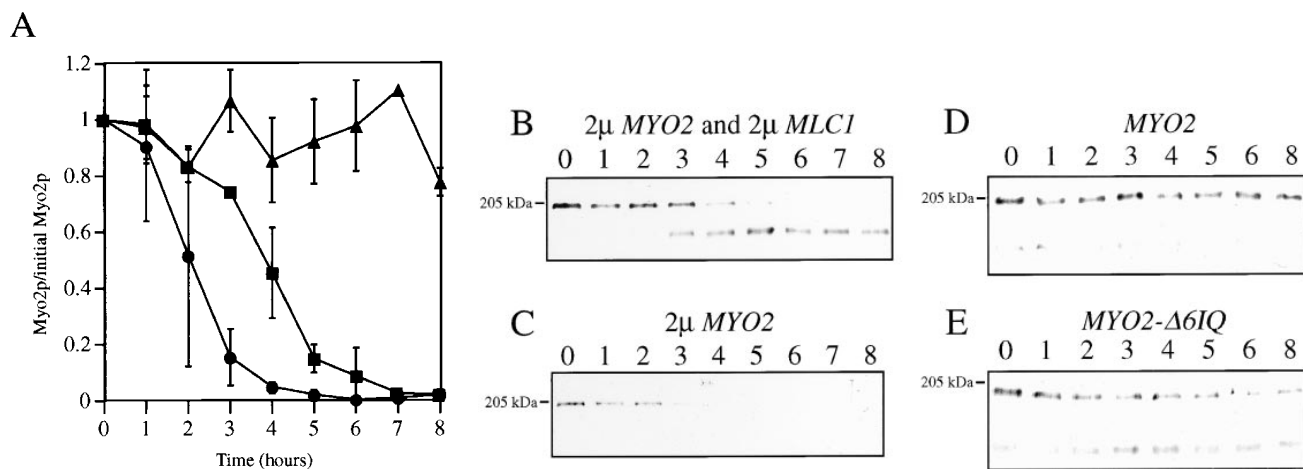


Figure 10. Degradation of Myo2p. Cycloheximide was added to a final concentration of 100 $\mu\text{g/ml}$ to log-phase cultures. Cultures were allowed to continue shaking, and samples were collected for immunoblot analysis as described in Materials and Methods. CRY1 containing either no plasmids (\blacktriangle), plasmids pRS31 (high copies of *MYO2*) and pRS290 (high copies of *MLC1*) (\blacksquare), or pRS31 (high copies of *MYO2*) and pGF27 (vector alone) (\bullet) was examined. Bands from immunoblots probed with anti-Myo2p antibody were quantified, and values were normalized for loading using a Coomassie blue-stained band that demonstrated high stability (A). The error bars represent standard deviation between two (wild type), two (with high copies of *MYO2* and high copies of *MLC1*), or three (with high copies of *MYO2*) independent experiments. The immunoblots probed with anti-Myo2p antibody demonstrate Myo2p stability in CRY1 carrying either pRS31 and pRS290 (B) or pRS31 and pGF27 (C), and in CRY1 (*MYO2* strain) alone (D) or in RSY21 (*MYO2-Δ61Q* strain) alone (E). Numbers represent hours after cycloheximide addition.

in yeast (Epp and Chant, 1997; Lippincott and Li, 1998). Iqg1p contains five IQ sites that could act as binding sites for Mlc1p. We propose that one essential function of Mlc1p is to act as a light chain for IQGAP.

Calmodulin is an additional light chain for Myo2p. We have previously shown that calmodulin and Myo2p coimmunoprecipitate from yeast cell extracts and interact in vitro. A mutation that is in the actin-binding site of Myo2p and interferes with Myo2p function dramatically worsens the phenotype conferred by mutations in calmodulin (Brockerhoff et al., 1994). Here we show that the localization of calmodulin to sites of cell growth is predominately dependent on the IQ sites of Myo2p with only a small contribution by the other yeast class V myosin Myo4p. In a *MYO2-Δ61Q* strain lacking *MYO4*, calmodulin localizes to the spindle pole body and to fast moving patches on the cell surface. The protein target that binds calmodulin in these fast moving patches has yet to be identified. The IQGAP protein in yeast localizes to the actin ring at the bud neck during cytokinesis (Epp and Chant, 1997; Lippincott and Li, 1998). Because calmodulin localization to the bud neck during cytokinesis depends on Myo2p, either calmodulin does not bind to yeast IQGAP or calmodulin binds IQGAP at levels below detection.

Only one other noncalmodulin unconventional myosin light chain has been characterized. Biochemical studies identified M1CLC as a light chain for *Acanthamoeba* myosin IC (Wang et al., 1997). Mlc1p and M1CLC share 31% sequence identity, and both proteins share significant identity with other calmodulin/EF-hand superfamily members. The chicken class V myosin has at least three light chains, calmodulin and two additional uncharacterized small proteins that copurify with the myosin (Cheney et al., 1993). These two small proteins have yet to be identified.

In conclusion, we presented several lines of evidence that Myo2p has at least two light chains, calmodulin and a newly characterized protein, Mlc1p. Mlc1p regulates the stability of Myo2p by binding to the neck region. Our characterization of Mlc1p may aid in the identification of the additional light chains of the chicken class V myosins.

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