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-Δ6IQp, 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 (YGL106w) is an essential gene that exhibits haploinsufficiency. Reduced levels of MYO2 overcome the haploinsufficiency of MLC1. The mutant MYO2-Δ6IQ 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 (Brockerhoff 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 (Brockerhoff et al., 1994). Not only is calmodulin present at sites of polarized growth (Brockerhoff and Davis, 1992), but calmodulin and Myo2p have direct physical contact through the IQ sites in the neck of Myo2p (Brockerhoff 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
growth when overexpressed (Haarer et al., 1994). Myo4p localizes to the bud and is essential for the polarized distribution of the asymmetric 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 (myosin 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-AflII 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'-CTCGGCGCCATCAGTGAATCTTACG-CCTGTAATATCTGCTG-3') creating plasmid pRS27. In the plasmid pRS28, a 0.8-kb EcoRI fragment containing TRP1 (Davis et al., 1986) was cloned into the Smal site of pLI831 (Geiser et al., 1993) to make pRS37. The shuttle vector pRS50 contains a 2-μm origin from a 2.1-kb EcoRI fragment of YEplastigated into the NotI site of pRS37. In both plasmids, the 5′ overhangs were filled in with Klenow, and the plasmid shuffle used in this study required a plasmid encoding D6IQ. A 5.6-kb ClaI fragment of MYO2 was cloned into the Smal site of pRS37 (Geiser et al., 1993) to make pRS37. The shuttle vector pRS50 contains a 2-μm origin from the 2.1-kb EcoRI fragment of YEplastigated 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 pRS30 contains a 2-μm origin from a 2.1-kb EcoRI fragment of YEplastigated into the NotI site of pRS37. In both plasmids, the 5′ overhangs were filled in by treatment with the Klenow fragment of DNA polymerase.

### Table I. Plasmids Used in This Study

| Plasmid | Parent vector | Relevant markers and construction | Reference or source |
|---------|---------------|----------------------------------|---------------------|
| pBluescriptII KS (+) | amp' f1 origin | | Stratagene |
| pGEX-2T | Puc with gene encoding GST | | Pharmacia |
| pQE30 | Puc with 6XHis polylinker | Qiagen |
| pRS306 | URA3 f1 origin | (Sikorski and Hieter, 1989) |
| pRS315 | CEN6 ARSH4 LEU2 f1 origin | (Sikorski and Hieter, 1989) |
| pRS316 | CEN6 ARSH4 URA3 f1 origin | (Sikorski and Hieter, 1989) |
| pGF27 | pRS304 | 2-μm origin (YEp24 fragment) inserted at AatII site | G. Zhu |
| pGF29 | pRS306 | 2-μm origin (YEp24 fragment) inserted at AatII site | G. Zhu |
| pGS28 | pSB5 | amp' trc promoter, CMD1 | J. Geiser |
| pJP10-2B | YCp50 | URA3 MYO2 CEN4 ARS1 | (Johnston et al., 1991) |

* Unless otherwise stated, all markers from the parent plasmid are present in the new construct. MYO2::D6IQ 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 VGL108w.
3353 (American Type Culture Collection, Rockville, MD) was cloned into the calmodulin. Based on the work described in this paper, we have re-directed mutagenesis (Kunkel et al., 1987). Because of the nucleotide NAR1 (5′-GCAATAAGATGCATAATGGCCCATTTGATGATGATCCAGGAAGAG3′) results in the addition of a glycine between amino acid residues 786 and 787 as well as the mutation ST78A. The oligonucleotide NAR4 (5′-GAAATTGAAACAACTTGAAGGCCGGCCGAGCT-ATCCGTTAATC-3′) results in the addition of a glycine between amino acid residues 927 and 928. After mutating pRS78 using NAR1 and NAR4, the resulting plasmid was digested with NarI and recoloned using T4 DNA ligase. This mutant cassette was then ligated to BamHI and EcoRI-cut pRS43 to make the plasmid pRS172, which encodes a Myo2p with an 878G mutation and lacks only amino acids 788–927. This mutant was named MYO2-ΔIQ. Plasmid pRS221 has the 5.4-kb Asp718-SacI fragment containing MYO2-ΔIQ cloned into pGF2.

The plasmid pRS174 was created to integrate MYO2-ΔIQ into the genome of S. cerevisiae. pRS174 was made by ligating the 5.4-kb plasmid cassette encoding MYO2-ΔIQ 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-3533 (American Type Culture Collection, Rockville, MD) was cloned into the BamHI site of pBluescriptII KS1. The new plasmid is MLC1-FOC1, creating plasmid pRS276. Plasmid pRS282 is a derivative of pRS276, in which the 2.0-kb NcoI-NdeI 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 polynucleotide.

The plasmid to delete MLC1 precisely was made in a similar way to the myo2 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′-CTAAATTTGCAGTTCCGCACTCT-CGAAATCTTGTATATCTATGGC-3′), creating a plasmid 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 Mlc1p in Escherichia coli, was made in several steps. An Ncol site was placed at the first codon of MLC1 by oligonucleotide-directed mutagenesis using primer MLC1-NCOI (5′-GGCTCTGGTGGCTGCCATGGTTATTCTATATCTATATCTAT-3′) (Kunkel et al., 1987) to make pRS291. This mutagenesis also changed the SnaI site to SflI and the SflI site to NcoI. The SnaI-NcoI fragment was inserted into pRS291 with NcoI, filling in the NcoI fragment of DNA polymerase, digesting with SacI, and purifying the 0.8-kb fragment. Plasmid pRS296 was then made by digesting pRS30 (Qiagen, Inc., Chatsworth, CA) with BamHI, filling in the Klenow fragment of DNA polymerase, digesting with SacI, and ligating in the 0.8-kb MLC1 fragment.

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**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 BglII and ClaI transformed into RSY1. Because strains carrying ade1Δ-100 are histidine auxotrophs, ADE3 on a plasmid can be selected by histidine prototrophy (Venkataraman et al., 1986). RSY2 was transformed with pRS50 carrying ADE3 in MLC1. The strain RSY2-60B has MLC1 deleted from the genome and carries pRS50.

The mutant MYO2-ΔIQ 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-ΔIQ by PCR and by Southern blot analysis. RSY22 was mated to strain MYY28 carrying an integrated form of GFP-CDM1 (Moser et al., 1997) to make the diploid strain RSY33. A myo4Δ::URA3 construct (a gift from Susan Brown, University of Michigan, Ann Arbor, MI) was transformed into RSY33 to create strain RSY38, heterozygous for GFP-CDM1.

**Strain RSY105**

Strain RSY105 carrying 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 diploid strain was made homozygous by mating and transformation with plasmid pRS286. The resulting strain still had two copies of the gene. The 1.7-kb BamHI-NdeI fragment of plasmid pRS286 encoding mlc1Δ::TRPI was integrated 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′-fluorodeoxyuridine (5′-FOA). One of these four colonies was the likely candidate for heterozygous mlc1Δ 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/mlc1Δ::TRPI by Southern blot analysis. RSY105 must carry a plasmid source of MLC1 to survive.

To study the null phenotype of mlc1Δ, strains RSY105(pRS289) and JGY46 were sporulated on Sp0II 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 0.4×–20× magnification. All colonies were transferred to 20 μl 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 μg/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 on 50 Klett units in the appropriate medium. After centrifugation, cells were washed in lysis buffer (50 mM Tris, pH 8.0, 1× 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 μg 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 blotted 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 (Amer sham 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 μg/ml thiamine, 50 μg/ml ampicillin, 15 μg/ml kanamycin, and 8 μCi 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 μl 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

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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     | MATα ade2-1 oc ade1-100 hist3-11,15 leu2-3,112 trpl-1 ura3-1 | This study               |
| JGY46    | MATa/MATa ade2-1 oc ade1-100 hist3-11,15 leu2-3,112 trpl-1 trpl-1 ura3-1 | This study               |
| EMY55-5D | MATa ade2-1 oc ade3-100 can1-100 cyc2 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| EMY55-6B | MATa ade2-1 oc ade3-100 can1-100 cyc2 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| MMY28    | MATa ade2-1 oc ade3-100 can1-100 (S65T-GFP)-CMD1 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY1     | EMY55-5D X EMY55-6B | This study               |
| RSY2     | MATa/MATa ade2-1 oc ade1-100 ade3-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY38    | MATa ade2-1 oc ade3-100 can1-100 cyc2 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY38-9D | MATa ade2-1 oc ade3-100 can1-100 GFP-CMD1 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY38-16A| MATa ade2-1 oc ade3-100 can1-100 GFP-CMD1 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY38-16C| MATa ade2-1 oc ade3-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY38-17C| MATa ade2-1 oc ade3-100 can1-100 GFP-CMD1 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY105   | MATa ade2-1 oc ade3-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY105-6A| MATa ade2-1 oc ade3-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::his3 trpl-1 ura3-1 | This study               |
| RSY107   | RSY21 X RSY105-6A carrying plasmid pRS289 | This study               |
| RSY112   | MATa/MATa ade2-1 oc ade3-100 ade3-1 can1-100 can1-100 his3-11,15,115 leu2-3,112 lys2Δ::his3 trpl-1 trpl-1 ura3-1 | 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 g for 30 min in a 4°C microcentrifuge. The supernatant fraction was batch mixed for 10 min with 700 μl 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 with 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 35S-labeled 6XHis-Mlc1p had a specific activity of 27 dpm/fmol.

Purified 35S-labeled calmodulin was made as previously described (Brockerhoff et al., 1992), except that only 8 mCi Na35SO4 was used in the labeling. The purified 35S-labeled 6XHis-Mlc1p had a specific activity of 39 dpm/fmol.

The gel overlay assays for calmodulin and Mlc1p binding were performed as previously described (Brockerhoff et al., 1994) with slight modifications. In brief, extracts were made from the E. coli strain GM1 (Cour- londre and Miller, 1977) containing either plasmid pGEX-2T, pSB20, pSB21, pSB24, pSB25, or pSB27. Approximately 30 μg 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, pH 7.2, 100 mM NaCl) and blocked for 6 h in buffer A containing 5% bovine serum albumin and 0.05% Tween-20. The membranes were probed for 16 h at 21°C with either 140 mM 35S-labeled 6XHis-Mlc1p or 156 mM 35S-labeled calmodulin in buffer C (buffer A with 5 mM CaCl2 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 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, ~7.0 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 μg 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 μg mRNA was loaded in each lane of a prerun formaldehyde gel. After removing the formaldehyde by washing 2× in DEPC H2O, 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 pRS22 and a PCR product containing a 0.5-kb fragment of TRX2. After incubating the membrane with ~1×106 cpm of each probe (pooled) for 48 h, the membrane was washed and analyzed using a Molecular Dynamics PhosphorImager (model 400B, 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 neckless Myo2p 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 1](image1.png)

**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).

![Figure 2](image2.png)

**Figure 2.** Comparison of the localization of Myo2p and Myo2p-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.
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

| Genotype | Polarized calmodulin (%) |
|----------|--------------------------|
| Wild type       | 84                      |
| myo2-Δ6IQ     | 15                      |
| myo4Δ          | 80                      |
| myo4Δ myo2-Δ6IQ | 4                       |

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 μm.

Table III. Calmodulin Localization in Myosin Mutants
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 1C 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.

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**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 1C light chain (*A. castellanii*, MLCIC) 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.

**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.
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.

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. 8B). As a control, we show calmodulin also binds specifically to fusion proteins that contain IQ sites (Fig. 8C). Both calmodulin and 6XHis-Mlc1p bind equally well in the presence of Ca²⁺ 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. 9A). 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. 9B). 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).
We hypothesized that high levels of calmodulin or Mlc1p would counteract the toxicity of overexpressed MYO2. Overexpressing \textit{CMD1} is unable to overcome the decreased growth rate (Fig. 9 \textit{A}) or the low levels of Myo2p (Fig. 9 \textit{D}) caused by overexpressed \textit{MYO2}. In contrast, a high-copy number plasmid carrying \textit{MLC1} is able to fully suppress the overexpression \textit{MYO2} defects and results in a strain that has high levels of Myo2p (Fig. 9, \textit{A} and \textit{C}). These results indicate that the neck region of \textit{MYO2} is indeed responsible for an overexpression growth defect and that this toxicity is overcome by high levels of Mlc1p.

\textbf{Mlc1p Stabilizes Myo2p}

Because high copies of \textit{MLC1} are able to overcome defects caused by overexpression of \textit{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 \textit{MYO2-D\textit{\textgamma}6IQ}, the Myo2-D\textit{\textgamma}6IQp has a half-life of greater than 8 h. The neckless Myo2-D\textit{\textgamma}6IQp 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 \textit{MYO2} and carrying high copies of \textit{MLC1}, the half-life of Myo2p is 4 h. High copies of \textit{MLC1} are able to stabilize Myo2p, thus suggesting Mlc1p is a light chain responsible for stabilizing the neck region of Myo2p.

\textbf{Discussion}

Electron micrographs of the chicken myosin V molecule show two heads connected to a 30-nm stalk. At the proximal 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 \alpha-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 \textit{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 \textit{Dictyostelium} myosin II heavy chain \textit{\textgamma}BLCBS, which lacks the two IQ sites, maintains 20% wild-type motor activity when measured in the sliding filament assay (Uyeda et al., 1996). The \textit{\textgamma}BLCBS myosin allows cell division in vivo but demonstrates slightly slowed furrow formation during cytokinesis (Zang et al., 1997). Thus, both Myo2-D\textit{\textgamma}6IQp and the \textit{\textgamma}BLCBS myosin confer relatively minor defects. These results indicate that the full efficiency of the myosin is not essential for myosin function in vivo.

\textit{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
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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-Δ6IQp 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. Inviable mlc1Δ 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.
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 Myo2Δ6IQ 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 MLC1C as a light chain for Acanthamoeba myosin IC (Wang et al., 1997). Mlc1p and MLC1C 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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