The Unconventional Myosin, Myo2p, Is a Calmodulin Target at Sites of Cell Growth in *Saccharomyces cerevisiae*

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Abstract. Myo2p is an unconventional myosin required for polarized growth in *Saccharomyces cerevisiae*. Four lines of evidence suggest that (*a*) Myo2p is a target of calmodulin at sites of cell growth, and (*b*) the interaction between Myo2p and calmodulin is Ca^{2+} independent. First, as assessed by indirect immunofluorescence, the distributions of Myo2p and calmodulin are nearly indistinguishable throughout the cell cycle. Second, a genetic analysis indicates that mutations in *CMD1* show allele-specific synthetic lethality with the *myo2-66* conditional mutation. Mutations that inactivate the Ca^{2+}-binding sites of calmodulin have little or no effect on strains carrying *myo2-66*, whereas an allele with a mutation outside the Ca^{2+}-binding sites dramatically increases the severity of the phenotype conferred by *myo2-66*. Third, Myo2p coimmunoprecipitates with calmodulin in the presence of Ca^{2+} or EGTA. Finally, we used a modified gel overlay assay to demonstrate direct interaction between calmodulin and fusion proteins containing portions of Myo2p. Calmodulin binds specifically to the region of Myo2p containing six tandem repeats of a motif called an IQ site. Binding occurs in either Ca^{2+} or EGTA, and only two sites are required to observe binding.

ESTABLISHING and maintaining cell polarity is of fundamental importance in biology. Neuron function, embryo development, cell movement, and yeast cell growth all depend on an asymmetric distribution of cellular components. Within most nerve cells, information, in the form of electrical activity, flows in a predictable and consistent direction from the dendrites and cell body along the axon to presynaptic release sites in the axon terminal (Kandel et al., 1991). Synaptic vesicles move along precisely organized cytoskeletal filaments to their appropriate destinations (for review see Portier, 1992). During development, the distribution of yolk protein within the egg cytoplasm influences the pattern of embryonic cleavage and defines the animal and vegetal poles (Gilbert, 1988). In the budding yeast, *Saccharomyces cerevisiae*, bud site position and the direction of bud growth are defined by the polarized arrangement of the actin cytoskeleton (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Unlike in more complicated organisms, it is possible in yeast to genetically dissect the pathways that lead to the development of cell polarity and thereby define the molecular mechanisms underlying this process.

Budding in *S. cerevisiae* consists of several steps: bud site selection, bud site assembly, cytoskeleton organization, and polarized growth. Proper bud site selection requires the function of all five *BUD* genes. The bud site assembly genes, *CDC24*, *CDC42*, *CDC43*, *BEM1*, and *BEM2*, are needed to assemble cytoskeletal components at the bud site to direct future bud formation and growth. Bud growth occurs by the localized fusion of secretory vesicles with the cell surface. Secretory vesicles are thought to be transported on an asymmetrically arranged actin-based cytoskeleton which extends from the mother cell towards the bud (for review see Drubin, 1991; Madden et al., 1992). A recently identified unconventional myosin, Myo2p, is a putative motor protein implicated in transporting vesicles to the bud along actin cables (Johnston et al., 1991).

Our recent studies indicate that calmodulin is also required for polarized growth in yeast. Under nonpermissive conditions, a temperature-sensitive calmodulin mutant is delayed in bud emergence and bud growth although RNA and DNA synthesis proceed at normal rates (Davis, 1992). The accumulation of calmodulin at sites of cell growth also implicates calmodulin in polarized growth (Brockerhoff and Davis, 1992). Indirect immunofluorescence studies revealed that calmodulin and actin distributions overlap throughout the cell cycle. Calmodulin concentrates at the presumptive bud site in an unbudded cell, at the tip in a growing bud, and in the neck region between mother and bud during cytokinesis. Calmodulin also accumulates at the shmoo tip in cells treated with mating pheromone. Interestingly, calmodulins mutated in the Ca^{2+}-binding sites support bud growth and localize similarly to wild-type calmodulin. Thus, the function of calmodulin during polarized growth does not depend on a high affinity for Ca^{2+}.

This study investigates the potential physical interaction

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between Myo2p and calmodulin at sites of cell growth. *MYO2* was identified in a genetic screen for yeast cells that increase in mass in the absence of continued proliferation. A strain containing the temperature-sensitive mutation *myo2-66* accumulates secretory vesicles and arrests as a large unbudded cell with chitin randomly distributed on its surface (Johnston et al., 1991). Furthermore, Myo2p concentrates at sites of cell growth (Lillie, S. H., and S. S. Brown. 1992. *Mol. Biol. Cell.* 3s:42a). Myo2p shares structural features with an unconventional myosin from vertebrate brain, p190, that binds to calmodulin in the presence of Ca^{2+} or EGTA (Espreafico et al., 1992). Like the brain protein, Myo2p has six tandem repeats of a Ca^{2+}-independent calmodulin-binding site (Johnston et al., 1991) known as an IQ site because its consensus sequence is IQXXXRGXXXR (for review see Cheney and Mooseker, 1992). The calmodulin-binding domains of p190 map precisely to the region containing this motif (Espreafico et al., 1992).

Materials and Methods

Media and Strains

Media for growth of *S. cerevisiae* and *Escherichia coli* are as described (Geiser et al., 1991; Davis, 1992). Genetic manipulations and yeast transformations were performed using standard techniques (Sherman et al., 1986). The *S. cerevisiae* strains are listed in Table I. Strains CRY1 and CRY2 (Davis, 1992) are derivatives of strain W303 (Wallis et al., 1989). All strains except strain JP7A are derived from strains CRY1 and CRY2. Strain JP7A (Johnston et al., 1991) contains a temperature-sensitive mutation in the *MYO2* gene, *myo2-66*, and was kindly provided by G. Johnston (Dalhousie University, Halifax, Nova Scotia). In order to reduce heterogeneity due to strain background differences, strain JP7A was backcrossed four times with CRY strains to produce strain SBY8. SBY8 was sporulated and haploid progeny containing *myo2-66* were identified by their inability to grow at 36°C.

Strains containing mutant forms of calmodulin were constructed as follows. Strain JGY41 (Geiser et al., 1991) was crossed with strain EMY55-2A (Muller, E., unpublished results) to produce EMY80. Strain EMY80 was sporulated and strain EMY80-43C containing *cmdl-3* was identified by its inability to grow at 37°C. The calmodulin gene, *cmdl-3*, encodes calmodulin with the mutations D20A, E31V, D56A, E67V, D93A, and E104V (Geiser et al., 1991). Strains JGY148, JGY149, JGY134, and BCY4 are CRY1 in which the wild-type calmodulin gene was replaced by the mutant calmodulin genes *cmdl-5*, *cmdl-6*, *cmdl-7*, and *cmdl-8* as described (Geiser et al., 1991). *cmdl-5* encodes calmodulin with the mutations E31V, E67V, and E104V (3E→V). *cmdl-6* encodes calmodulin with the mutations D20A, D56A, and D93A (3D→A). The mutant calmodulin encoded by *cmdl-7* has isoleucine 27 changed to asparagine (I27N) and leucine 71 changed to arginine (L71R). *cmdl-8* encodes calmodulin in which glycine in position 113 is replaced with a valine (G113V).

Strain SBY21 containing a deletion of the *SPA2* gene was constructed as follows. CRY1 was transformed with a 3.8-kb SalI-HindIII fragment from plasmid p210 (Gehrung and Snyder, 1990) and Ura^+ transformants were selected. In plasmid p210, a 3,500-bp segment of *SPA2* is replaced with the *URA3* gene; the mutant *SPA2* gene contains 39 codons of *SPA2* upstream of *URA3*. The presence of the deletion was confirmed by Southern blot analysis (data not shown).

Plasmids

Seven fusions of *MYO2* with glutathione *S*-transferase (GST)[1] were made using the GST Gene Fusion System (Pharmacia Diagnostics Inc., Fairfield,

Table I. Yeast Strains

Strain	Genotype	Reference
CRY1	*MAT***a** *ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Fuller (Stanford University, Stanford, CA)
CRY2	*MATα ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Fuller
TDY62-13A	*MATα ade2-1oc ade3Δ-100 can1-100 cmd1-1 leu2-3,112 trp1-1 ura3-1*	T. N. Davis, 1992
JGY41	*MAT***a** *ade2-1oc can1-100 cmd1-3 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. R. Geiser et al., 1991
JP7A	*MAT***a** *ade1 his6 leu2-3,112 ura3-52 myo2-66*	G. C. Johnston, 1991
EMY55-2A	*MATα ade2-1oc ade3Δ-100 can1-100 cyh2^r his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 trx1Δ::TRP1 ura3-1*	E. Muller (University of Washington, Seattle, WA)
EMY80	EMY55-2A X JGY41	E. Muller
EMY80-43C	*MAT***a** *ade2-1oc ade3Δ-100 can1-100 cmd1-3 his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1*	E. Muller
JGY134	*MAT***a** *ade2-1oc can1-100 cmd1-7 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. R. Geiser (University of Washington, Seattle, WA)
JGY148	*MAT***a** *ade2-1oc can1-100 cmd1-5 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. R. Geiser
JGY149	*MAT***a** *ade2-1oc can1-100 cmd1-6 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. R. Geiser
BCY4	*MAT***a** *ade2-1oc can1-100 cmd1-8 his3-11,15 leu2-3,112 trp1-1 ura3-1*	B. Chang (University of Washington, Seattle, WA)
SBY8-5A	*MATα ade2-1oc can1-100 his$^-$ leu2-3,112 myo2-66 ura3$^-$*	This study
SBY8-6A	*MAT***a** *ade2-1oc can1-100 his$^-$ leu2-3,112 myo2-66 ura3$^-$*	This study
SBY11	TDY62-13A X SBY8-6A	This study
SBY13	SBY8-5A X EMY80-43C	This study
SBY14	SBY8-5A X JGY134	This study
SBY14-19A	*MAT***a** *ade2-1oc can1-100 cmd1-7 his$^-$ leu2-3,112 myo2-66 ura3$^-$*	This study
SBY14-20C	*MAT***a** *ade2-1oc can1-100 cmdl-7 his$^-$ leu2-3,112 myo2-66 trp1-1 ura3$^-$*	This study
SBY15	SBY8-5A X JGY148	This study
SBY16	SBY8-5A X JGY149	This study
SBY17	SBY8-5A X BCY4	This study
SBY21	*MAT***a** *ade2-1oc can1-100 his3-11,15 leu2-3,112 spa2Δ::URA3 trp1-1 ura3-1*	This study
SBY23	TDY62-13A X SBY21	This study
SBY23-2A	*MAT***a** *ade2-1oc ade3Δ-100 can1-100 cmdl-1 his3-11,15 leu2-3,112 spa2Δ::URA3 trp1-1 ura3-1*	This study

NJ) (see Fig. 4). Fragments of *MYO2* were obtained from plasmid pJP10-2B (Johnston et al., 1991).

In plasmid **pSB20**, the nucleotides encoding amino acid residues 740–1,457 of Myo2p were fused in frame to GST by ligating the 2.1-kb EcoRV fragment of *MYO2*, into the SmaI site of pGEX-3X (Pharmacia Diagnostics Inc.). Plasmid **pSB21** contains the 1.1-kb EcoRV-EcoRI *MYO2* fragment fused in frame to GST in plasmid pGEX-3X and was constructed by digesting pSB20 with EcoRI and then religating. The EcoRV-EcoRI fragment encodes residues 740–1,116 of Myo2p. To create the next construct, plasmid pSB21, grown in a dam$^-$ *E. coli* strain GM2163 (NEB), was linearized with EcoRI and then partially digested with BclI. A 5.4-kb fragment obtained from the partial digestion was gel purified, treated with the large fragment of *E. coli* DNA polymerase in the presence of dNTPs, and then religated. The resulting plasmid, **pSB23**, contains a 0.5-kb EcoRV-BclI fragment encoding amino acid residues 740–912 of Myo2p. Plasmid **pSB24** contains a 0.4-kb fragment of *MYO2* encoding residues 790–924 fused in frame to GST in pGEX-3X. This *MYO2* fragment was obtained using PCR. The primers used were:

SB-3: 5′-GCTCGGATCCAGAAGAAAATTAGAGCTAAATATTAC-3′
and
SB-4: 5′-CGCGGATCCTTTCAATTTCCTTTGAGCAGCTCTTCT-3′.

Each primer contains a BamHI site (GGATCC) near the 5′ end to facilitate subcloning. Approximately 1 ng of linearized plasmid pJP10-2B was used in each 50 μl PCR. The final concentration of each primer was 1 μM. Reagents for PCR were supplied by Gene-Amp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, CT). After amplification, four-fifths of the reaction was run on a 1% agarose gel and purified. The purified *MYO2* fragment was then digested with BamHI and ligated into the BamHI site of plasmid pGEX-3X. Plasmid **pSB25** was constructed by first digesting pSB21 with EcoRV and PvuII and religating to create plasmid pSB21A. Plasmid pSB21A was then digested with HpaI and EcoRI, treated with the large fragment of *E. coli* DNA polymerase in the presence of dNTPs, and then religated. The resulting plasmid, pSB25, contains a 279-bp EcoRV-HpaI *MYO2* fragment fused in frame to GST. The EcoRV-HpaI *MYO2* fragment encodes residues 740–833. Plasmid **pSB26** was constructed by digesting plasmid pSB21A with EcoRI and NsiI and then religating. Plasmid pSB26 contains a 129-bp *MYO2* fragment encoding residues 740–782. Finally, in plasmid **pSB27**, the nucleotides encoding amino acid residues 247–740 of Myo2p were fused in frame to GST by ligating a 1.5-kb BamHI-EcoRV fragment of *MYO2* into the BamHI-SmaI sites of plasmid pGEX-3X.

Plasmid pJG58 is a derivative of plasmid YEp24 and was constructed by replacing the BamHI-SalI fragment of pTD17 (Zhu et al., 1993) with a BamHI-SalI fragment containing *cmdl-3* from plasmid pJG27 (Geiser et al., 1991).

Fusion Protein Expression

E. coli strain GM1 (Coulondre and Miller, 1977) containing either plasmid pGEX-3X, pSB20, pSB21, pSB23, pSB24, pSB25, pSB26, or pSB27 was grown in LB medium containing 100 μg/ml ampicillin. At approximately 15 Klett units (7.5×10^7 cells/ml), isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 2 mM. At 80 Klett units, cultures were harvested by centrifugation and then resuspended in 1/100 volume "cracking" buffer containing 0.01 M sodium phosphate, pH 7.2, 1% β-mercaptoethanol, 1% SDS, and 6 M urea. Although growth to higher densities was possible, the amount of fusion protein obtained was significantly reduced in denser cultures. After incubation for 30 min at 37°C in cracking buffer, one volume 2× Laemmli sample buffer was added. SDS-polyacrylamide gel electrophoresis was done as described (Brockerhoff et al., 1992). The acrylamide concentration was 11% and the ratio of acrylamide to bisacrylamide was 39:1. Approximately 30 μg total protein was loaded in each lane. The gel overlay assay for calmodulin-binding proteins was performed as described (Brockerhoff et al., 1992) with the following changes. After transfer to the Immobilon membrane, proteins were renatured by washing for 30 min (three changes) in 20 mM Hepes, pH 7.2, and then the membrane was blocked overnight in 20 mM Hepes, pH 7.2, with 0.05% Tween-20 and 3% BSA. Membranes were probed for 1–2 h in 20 mM Hepes, pH 7.2, 10 mM NaCl, 0.1% BSA containing either 1 mM EGTA or 1 mM $CaCl_2$, and 80 nM ^{35}S-labeled wild-type calmodulin.

Extraction of Myo2p and Immunoprecipitation

Myo2p was extracted from yeast cells in a buffer containing 0.5 M NaCl as follows. A 200-ml yeast culture was grown in YPD (Geiser et al., 1991) at 30°C to 150 Klett units (3×10^7 cells/ml). Cells were harvested and washed once with buffer A (50 mM Tris, pH 7.5, and protease inhibitor cocktail [Drubin et al., 1988]) and then resuspended in an equal volume of buffer A. Cells were lysed in the presence of one volume glass beads in a plastic tube by mixing five times for 1 min on a vortex mixer. Myo2p is insoluble in buffer A and was collected by centrifugation for 15 min at 4°C in a microfuge (Fisher model 235C, Pittsburgh, PA). The pellet was washed once with 0.7 ml buffer A. Then ~50% of the Myo2p was extracted with buffer A containing 0.5 M NaCl. The sample was incubated on ice for 5 min, and insoluble debris was removed by centrifugation in the microfuge at 4°C for 15 min. If the extracts were prepared in glass tubes or in a bead beater, the yield of Myo2p was dramatically decreased. $CaCl_2$ (5 mM) or EGTA (1 mM) was added to the supernatants. Approximately 250 μg total protein (100 μl of the high-salt supernatant) was incubated with an aliquot (10 μl) of anti-calmodulin antiserum, affinity-purified anti-calmodulin antibody, or preimmune serum from the same rabbit for 60 min on ice. An aliquot (50 μl) of pansorbin cells (Calbiochem Novabiochem, Corp., La Jolla, CA) was added to each sample and the incubation continued for 60 min. After centrifugation in the microfuge at 4°C for 2 min, the pellets were washed once with 100 μl buffer A containing 0.5 M NaCl and then resuspended in 30 μl Laemmli buffer. The supernatants were precipitated with trichloroacetic acid and resuspended in Laemmli sample buffer (30 μl). Samples were boiled for 3 min before SDS-gel electrophoresis.

Antibodies and Immunoblot Analysis

The calmodulin antiserum and antibodies were described previously (Brockerhoff and Davis, 1992). The anti-Myo2p antibody was affinity purified as described (Lillie and Brown, 1987) using a β-galactosidase-Myo2p fusion protein immobilized on nitrocellulose. Immobilized fusion protein and anti-Myo2p antiserum (prepared against an anthranilate synthase-Myo2p fusion) was kindly provided by S. Brown and S. Lillie (University of Michigan, Ann Arbor, MI). Extensive experiments establishing the specificity of the anti-Myo2p antibody will be presented elsewhere (Lillie, S. H., and S. S. Brown, manuscript in preparation).

Immunoblot analysis was done using ECL reagents (Amersham Corp., Arlington Heights, IL) as described (Geiser et al., 1991) with the following exceptions. For detection of calmodulin, the membrane was incubated with a 1:2,000 dilution of anti-calmodulin serum overnight and subsequently incubated with a 1:5,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase. For analysis of Myo2p, the acrylamide concentration in the gel was 6% and the ratio of acrylamide to bisacrylamide was 39:1. The membrane was incubated with a 1:100 dilution of affinity-purified anti-Myo2p antibody overnight.

Genetic Analysis

Six different alleles of *CMD1* were tested for their interaction with the temperature-sensitive allele *myo2-66* by comparing the phenotypes of strains containing mutations in both *CMD1* and *MYO2* to the phenotype of strains containing mutations in either *MYO2* or *CMD1*. We obtained the double mutant strains by crossing a strain containing a mutant calmodulin, with a strain containing *myo2-66* (Table I) and then sporulating the heterozygous diploid. We determined whether a spore had one or both single mutations by examining spore growth at 21, 25, 30, 32, 34, and 37°C (Table II). We concluded that the double mutant phenotype was the same as the more severe single mutant when >90% of the tetrads had four viable spores, none of which died at a lower temperature than the parent strains. In this case a parental ditype (PD) had four temperature-sensitive (ts) spores, a tetratype (TT) had three ts spores and one wild-type, and a nonparental ditype (NPD) had two ts spores and two wild-type spores. We concluded that the double mutant was inviable at room temperature if many tetrads had only two or three viable spores and 0/2 (NPD) or 2/3 (TT) spores were temperature sensitive.

Results

Calmodulin and Myo2p Have Similar Distributions

Lillie and Brown (1992, *Mol. Biol. Cell.* 3s:42a) demon-

1. *Abbreviations used in this paper*: GST, glutathione S-transferase; ts, temperature sensitive.

Table II. Genetic Interactions between Mutant Forms of CMD1 and myo2-66

CaM mutations	Death temperature (*cmd1* alone)	Death temperature (*cmd1*, *myo2-66*)
	°C	°C
None (*CMD1*)	39–40	32
I100N, E104V (*cmd1-1*)	32	<21
127N, L71R (*cmd1-7*)	37	30
G113V (*cmd1-8*)	38	<21
3E→V,3D→A (*cmd1-3*)	37	30
3E→V (*cmd1-5*)	39–40	30
3D→A (*cmd1-6*)	39–40	32

Strains containing only *myo2-66* are dead at 32°C (top row).

strated that Myo2p localizes at sites of cell growth throughout the cell cycle. We did a side by side comparison of Myo2p and calmodulin and found that the distribution of the two proteins is nearly identical (Fig. 1). Unbudded cells contained a patch of Myo2p. Small budded cells had Myo2p throughout the bud. Medium budded cells had Myo2p concentrated more towards the bud tip, and large budded cells had Myo2p concentrated in the neck region between mother and bud (Fig. 1). Furthermore, the region containing Myo2p was similar in size to the region containing calmodulin. These results are consistent with calmodulin and Myo2p interacting at sites of cell growth. Two minor differences in the distributions were observed. Calmodulin appeared more diffusely distributed throughout the cell than Myo2p and the mother cell was more brightly stained with calmodulin antibody than with Myo2p antibody consistent with calmodulin being required in processes in addition to polarized growth (Davis, 1992).

CMD1 Interacts Genetically with MYO2 but not with SPA2

Next, we used genetic methods to determine if *CMD1* and *MYO2* interact. We compared the phenotype of strains containing mutations in both *CMD1* and *MYO2* to the phenotype of strains containing mutations in either *CMD1* or *MYO2*. The phenotype of some strains containing temperature-sensitive mutations in both *CMD1* and *MYO2* is more severe than the phenotype of strains carrying either mutation alone. The severity of the double mutant phenotype varies for different calmodulin alleles (Table II).

cmdl-1 was the first temperature-sensitive calmodulin allele isolated and is the best characterized. Strains containing *cmdl-1* show a delay in bud emergence and bud growth (Davis, 1992). A strain containing both *cmdl-1* and *myo2-66* was inviable at room temperature. Thus a *CMD1* mutant known to show a defect in polarized growth is inviable when combined with *myo2-66*.

Two less characterized *CMD1* mutants, *cmdl-7* and *cmdl-8*, also show a genetic interaction with *myo2-66*. Although strains containing only *cmdl-7* or only *myo2-66* grew at 30°C, the double-mutant strain containing both of these mutations did not grow at this temperature (Fig. 2). A more severe interaction was detected between the mutations *cmdl-8* and *myo2-66*. Strains containing these two mutations were inviable at room temperature (21°C). Interestingly, strains containing only *cmdl-7* or *cmdl-8* were inviable at nearly the same temperatures, 37 and 38°C, respectively. Thus, the severity of the double mutant phenotype does not correlate with the severity of the temperature-sensitive phenotype of a calmodulin mutant; strains containing both *cmdl-7* and *myo2-66* or both *cmdl-8* and *myo2-66* died at significantly different temperatures while strains containing only *cmdl-7* or *cmdl-8* died at approximately the same temperature.

To examine the interaction between calmodulin and Myo2p in more detail we examined the terminal morphology of a strain containing both *cmdl-7* and *myo2-66* after incubation at its nonpermissive temperature, 30°C. At this temperature, the double mutant strain accumulated large unbudded cells (data not shown). This terminal phenotype resembled that of the parent strain containing only *myo2-66* when incubated at 32°C or higher (Johnston et al., 1991; data not shown). In contrast, at 37°C or above, the terminal morphology of the strain containing only *cmdl-7* is heterogeneous (B. Chang, University of Washington, Seattle, WA, unpublished result). Thus mutations in calmodulin exacerbate the defect associated with a loss of Myo2p function.

Previously we demonstrated that strains containing mutant forms of calmodulin defective in binding Ca^{2+} grew at the same rate as strains containing wild-type calmodulin (Geiser et al., 1991). Furthermore, the distributions of calmodulin containing the mutations 3D→A or 3E→V (see Materials and Methods) were indistinguishable from the wild-type protein (Brockerhoff and Davis, 1992). Although the affinity of these mutant proteins for Ca^{2+} is decreased more than 100-fold, strains relying on the mutant calmodulins grow buds at the same rate as wild-type cells. As expected, the genes encoding 3D→A (*cmdl-6*) and 3E→V (*cmdl-5*) calmodulin displayed little interaction with *myo2-66*. Growth of strains with *cmdl-6* and *myo2-66* was indistinguishable at all temperatures from the growth of strains carrying *myo2-66* alone. The presence of *cmdl-5* decreased the death temperature of *myo2-66* strains by only 2°C (Table II). A calmodulin gene with six mutations in the Ca^{2+}-binding sites, *cmdl-3*, also decreased the death temperature by 2°C (Table II), but even at 21°C, strains carrying *cmdl-3* and *myo2-66* grew very slowly. The negative interaction between *cmdl-3* and *myo2-66* is largely explained by the low level of calmodulin produced by the *cmdl-3* allele (27% of the wild-type level) because extra copies of *cmdl-3* on a multi-copy plasmid, pJG58, allows the double mutant carrying *cmdl-3* and *myo2-66* to grow well up to 30°C.

In contrast to our results suggesting an interaction between calmodulin and Myo2p, several results suggest calmodulin does not interact with Spa2p, another protein found at sites of cell growth (Snyder, 1989; Gehrung and Snyder, 1990; Snyder et al., 1991). First, a double-mutant strain containing a deletion of *SPA2* and *cmdl-1* had the same phenotype as a strain containing *cmdl-1* alone, indicating that *SPA2* and *CMD1* do not interact genetically. Second, we performed a side by side comparison of cells stained with anti-Spa2p (gift

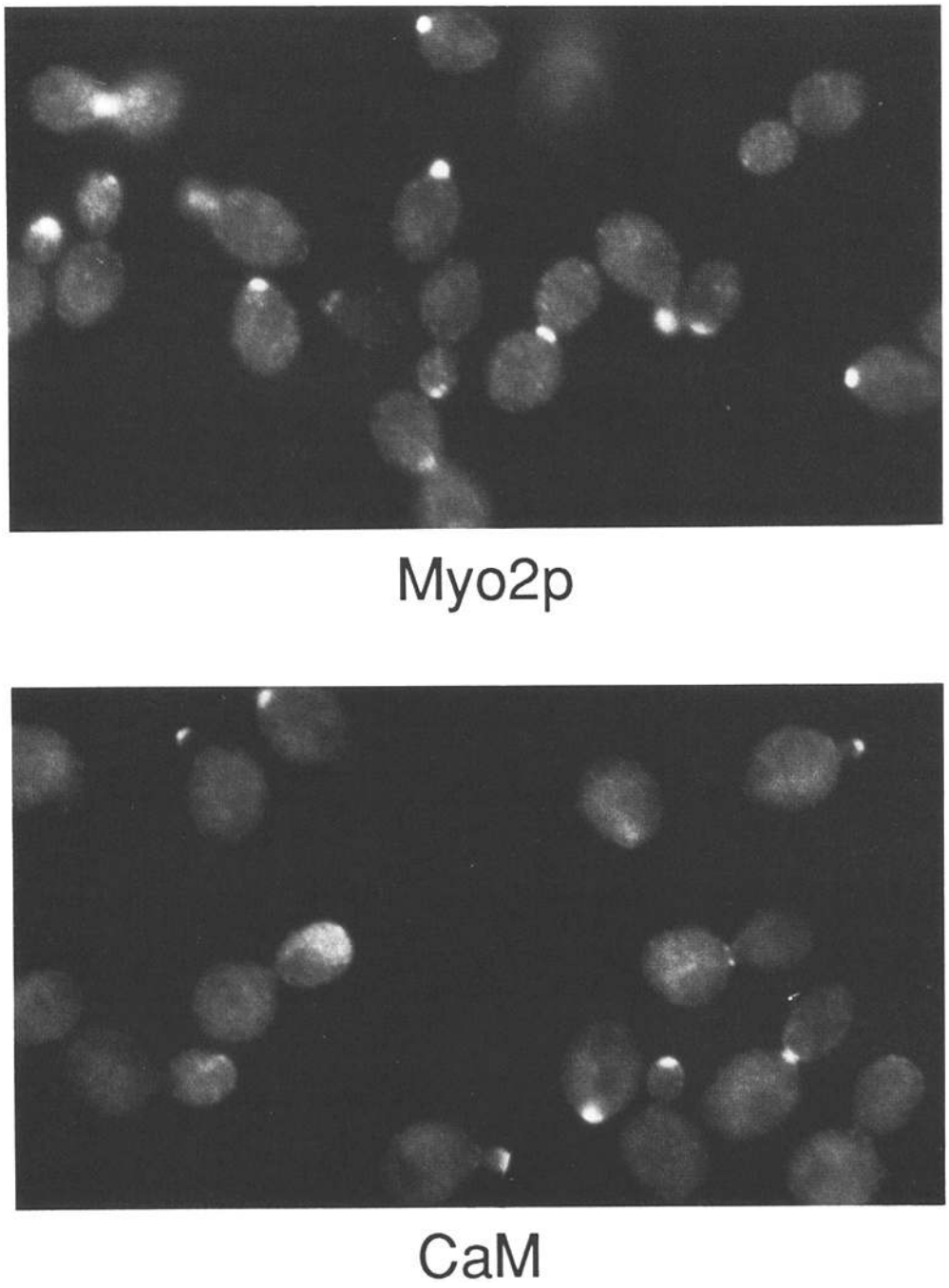

Figure 1. Comparison of the localization of Myo2p and calmodulin (*CaM*). Cells were stained with affinity-purified anti-yeast calmodulin antibody as described (Brockerhoff and Davis, 1992). The procedure to stain cells with anti-Myo2p antibody was the same as with anti-calmodulin antibody except cells were incubated with a 25-fold dilution of antibody overnight. Affinity-purified anti-Myo2p antibody was kindly provided by S. Brown and S. Lillie.

of M. Snyder, Yale University, New Haven, CT) to cells stained with anti-calmodulin and found that the distributions of the two proteins are similar but distinct. Spa2p is exclusively localized at the bud tip, whereas calmodulin and Myo2p are more diffuse (data not shown).

Calmodulin Associates with Native Myo2p

The immunocytochemistry and the genetic analysis suggest that Myo2p and calmodulin interact at sites of cell growth and that the interaction does not depend on a high affinity for Ca^{2+}. To obtain direct evidence for a molecular complex between calmodulin and Myo2p in yeast extracts, we analyzed calmodulin immune complexes for the presence of Myo2p. First we developed a method to solubilize yeast Myo2p using high salt (see Materials and Methods). Then the crude yeast extract containing solubilized Myo2p was immunoprecipitated with anti-calmodulin antiserum, affinity-purified anti-calmodulin antibody, or preimmune serum from the same rabbit. In 9 of 11 experiments, anti-calmodulin antiserum recovered Myo2p whereas the preimmune serum did not (Fig. 3). The recovery of Myo2p did not depend on the presence of Ca^{2+} since Myo2p was also precipitated when EGTA was present (Fig. 3). Furthermore, precipitation of calmodulin with affinity-purified antibody also recovered Myo2p (Fig.

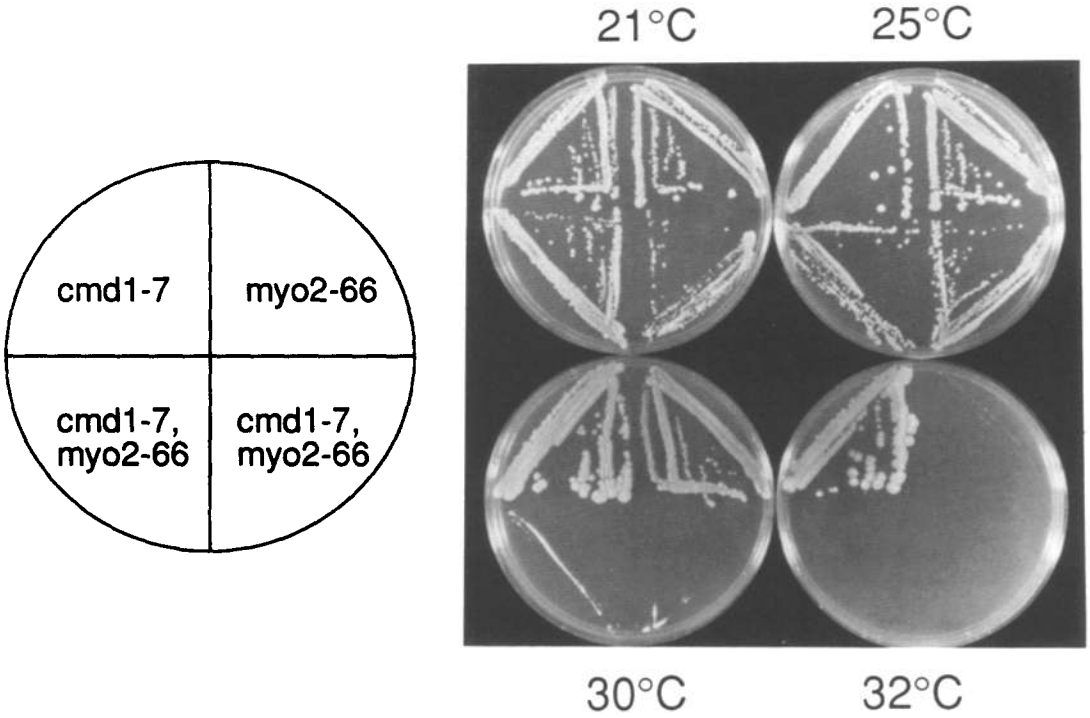

Figure 2. Growth of yeast strains containing *cmdl-7, myo2-66,* or both mutations. Strain JGY134 (*cmdl-7*), strain SBY8-5A (*myo2-66*), strain SBY14-19A (*cmdl-7, myo2-66*), and strain SBY14-20C (*cmdl-7, myo2-66*) were plated onto YPD plates and incubated at the indicated temperatures for 4 d. JGY134 (*cmdl-7*) is temperature-sensitive for growth at 37°C.

3). In two experiments Myo2p was not precipitated for unknown reasons. As an additional control for the specificity of the antibody, we demonstrated that anti-yeast calmodulin antibody does not recover Myo2p from a yeast strain expressing vertebrate calmodulin instead of yeast calmodulin (data not shown). (We have shown previously that our anti-calmodulin antiserum does not cross-react with vertebrate calmodulin [Brockerhoff and Davis, 1992]). These results demonstrate that calmodulin and Myo2p can form a molecular complex in 0.5 M NaCl, in the presence of Ca^{2+} or EGTA.

Calmodulin Interacts with Myo2p In Vitro

We used a modified gel overlay assay (Brockerhoff et al., 1992) to determine whether yeast calmodulin directly binds to the six putative IQ type calmodulin-binding sites in Myo2p. In this assay proteins are subjected to electrophoresis on an SDS gel and transferred to a membrane. The membrane is then washed to remove SDS and probed with radiolabeled calmodulin.

Portions of Myo2p were expressed in *E. coli* as a fusion with GST. The six IQ sites extend from residue 790–940. Seven different fusions containing portions of Myo2p extending from residue 247–1,457 were constructed (see Fig. 4). (Full-length Myo2p contains 1,574 residues.) The fusion proteins exhibited electrophoretic mobilities on SDS-PAGE consistent with their predicted size (Fig. 5 *A*). Calmodulin binding was mapped precisely to the residues between 790 and 940 (Fig. 5, *B* and *C*). Furthermore, fusions containing all six IQ sites interacted well with yeast calmodulin in either

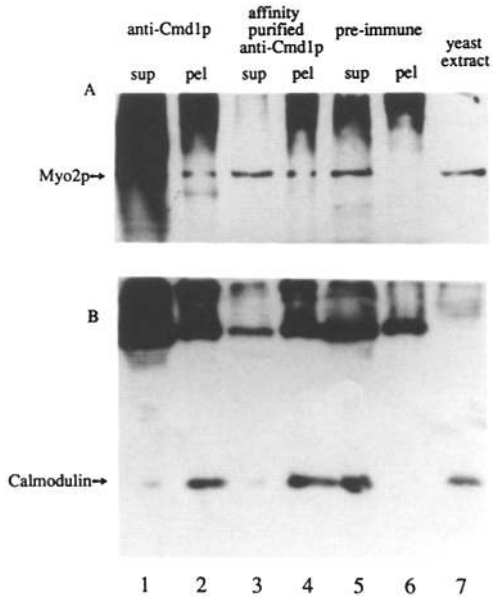

Figure 3. Immunoprecipitation with anti-calmodulin antisera. Yeast extracts containing solubilized Myo2p were incubated with anti-calmodulin antiserum (lanes *1* and *2*), affinity-purified anti-calmodulin antibodies (lanes *3* and *4*) or preimmune antiserum (lane *5* and *6*). Immunoprecipitations were performed in the presence of EGTA as described in Materials and Methods. Supernatants (lanes *1, 3,* and *5*) and precipitates (lanes *2, 4,* and *6*) were examined by anti-Myo2p (*A*) or anti-calmodulin (*B*) immunoblots as described in Materials and Methods.

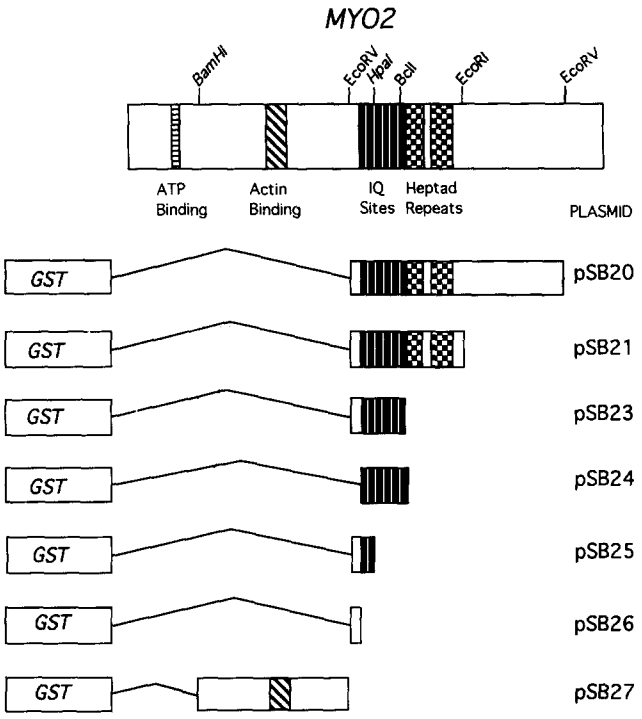

Figure 4. Myo2p fusion proteins. Plasmid construction is described in Materials and Methods. The indicated restriction sites were used for cloning. These sites are not necessarily unique (Johnston et al., 1991).

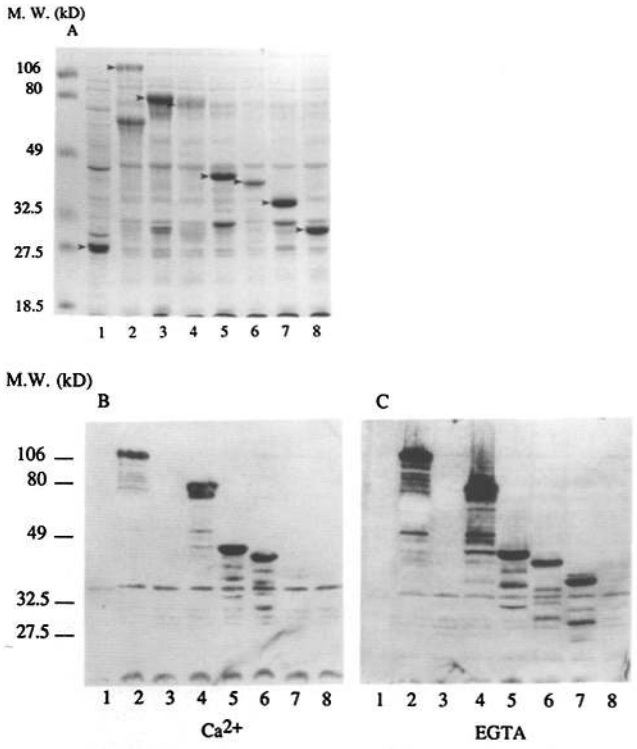

Figure 5. Identification of the IQ sites as the calmodulin-binding region of Myo2p. Fusion proteins were expressed and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. SDS gel stained with Coomassie blue R250 (*A*). The full-length fusion protein in each extract is marked by an arrow. Autoradiographs of membranes incubated with ^{35}S-labeled calmodulin in the presence of Ca^{2+} (*B*) or EGTA (*C*). The gel overlay assay was performed as described in Materials and Methods. Lanes *1–8* contain extracts of *E. coli* strain GM-1 containing plasmid pGEX-3X, pSB20, pSB27, pSB21, pSB23, pSB24, pSB25, and pSB26, respectively (see Fig. 4).

the presence of Ca^{2+} (Fig. 5 *B*) or EGTA (Fig. 5 *C*). We judged that the interaction between calmodulin and the various fusions was specific since other abundant proteins such as GST (lane *1*), and fusions containing Myo2p fragments lacking the IQ sites (lanes *3* and *8*), did not bind to calmodulin. Two IQ sites (lane *7*) are sufficient for calmodulin binding but unlike fusions containing all six IQ sites (lanes *2*, *4*, and *6*), the fusion containing two sites bound significantly better in the presence of EGTA than in the presence of Ca^{2+} (compare lane *7* in 5, *B* and *C*).

Discussion

The following results suggest that calmodulin participates in polarized cell growth by regulating Myo2p through a direct interaction. First, we detect a specific genetic interaction between *CMD1* and *MYO2*. Second, the localization of Myo2p and calmodulin are nearly indistinguishable. Third, calmodulin and Myo2p associate in yeast extracts. Finally, calmodulin binds specifically to the IQ sites of Myo2p in the presence of Ca^{2+} or EGTA in a modified gel overlay assay.

Myo2p is a recently identified member of the class V family of unconventional myosins (Johnston et al., 1991; Espreafico et al., 1992). The other members include p190 from vertebrate brain (Espreafico et al., 1992), *dilute* from mouse (Mercer et al., 1991), and Myo4p from yeast (EMBL/Genbank/DDBJ accession number M90057). With the exception of p190, members of this class have not been biochemically characterized. p190 is a calmodulin-binding protein associated with the actin-based cytoskeleton in brain. It is a phosphorylation substrate for calmodulin-dependent kinase II and has Mg^{2+}-ATPase activity that is stimulated severalfold by the addition of Ca^{2+} (Larson et al., 1990; Espindola et al., 1992). Calmodulin binds tightly to p190 and remains associated during gel filtration chromatography in the presence of Ca^{2+} or EGTA (Larson et al., 1988). Besides calmodulin, no other polypeptides in the 16–20-kD range remain associated with p190 (Espindola et al., 1992). Calmodulin also interacts with chicken brush border myosin I, a member of a different class of unconventional myosins (Mooseker et al., 1991). Since myosin light chains are members of the calmodulin/EF-hand superfamily, calmodulin may serve as regulatory light chains for a subclass of unconventional myosins (Cheney and Mooseker, 1992; Espreafico et al., 1992).

The site proposed to function as the light chain binding site in conventional and unconventional myosins is referred to as an IQ site and has the conserved core sequence IQXXXRGXXXR. All myosins contain one or more IQ sites (Cheney and Mooseker, 1992). The site of interaction of p190 with calmodulin has been precisely mapped to a region containing six imperfect tandem repeats of this motif (Es-

preafico et al., 1992). Several IQ repeats are also present in the region of brush border myosin I implicated in calmodulin binding (Carboni et al., 1988; Coluccio and Bretscher, 1988; Cheney and Mooseker, 1992). Like p190, Myo2p has six IQ repeats (Johnston et al., 1991). In this study we demonstrate that calmodulin associates with Myo2p and that the calmodulin-binding site in Myo2p maps exactly to the region containing the six IQ repeats. To determine if the combined presence of all sites is necessary for calmodulin binding, we constructed a fusion containing only the first two IQ sites. This two-site fusion protein also interacted with calmodulin indicating that at least one of the first two sites is active in binding calmodulin. The functional significance of several tandemly repeated calmodulin-binding sites in unconventional myosins is unclear. Using the genetic techniques available in *S. cerevisiae* we can now begin to mutate and delete the IQ sites in vivo to determine the role of each IQ site in polarized growth.

Calmodulin associates with Myo2p in the presence of Ca^{2+} or EGTA both in solution and in an overlay assay. Since most calmodulin-binding proteins bind calmodulin only in the presence of Ca^{2+} (Cohen and Klee, 1988) this Ca^{2+}-independent association suggests a unique mechanism of regulation. One proposal, based on studies with p190, is that calmodulin associates with p190 irrespective of Ca^{2+} and allosterically regulates ATPase activity through Ca^{2+}-dependent conformational changes. This hypothesis is based on the finding that the addition of Ca^{2+}-calmodulin stimulates the ATPase activity of p190 to a greater extent than Ca^{2+} alone (Larson et al., 1990; Espindola et al., 1992). In contrast, our results in yeast indicate that Ca^{2+} binding to calmodulin is not needed for normal bud growth. Mutant calmodulins defective in binding Ca^{2+} properly localize to sites of cell growth and strains relying on these mutant proteins do not show growth defects (Geiser et al., 1991; Brockerhoff and Davis, 1992). The results of the genetic analysis are also consistent with the idea that the ability of calmodulin to interact with or regulate Myo2p is not dependent on a high affinity for Ca^{2+}. Mutations in *CMD1* that abolish the ability of calmodulin to bind Ca^{2+} have little or no effect on strains carrying the temperature-sensitive mutation, *myo2-66*. In contrast, a severe negative interaction was observed between *myo2-66* and the allele *cmd1-8* with a mutation outside the Ca^{2+}-binding sites.

Calmodulin binding to Myo2p could be regulated by phosphorylation as has been proposed for other Ca^{2+}-independent calmodulin-binding proteins such as the neuron-specific protein kinase C substrates, neuromodulin, and neurogranin. Both proteins contain a protein kinase C phosphorylation site within an IQ type calmodulin-binding site. Protein kinase C phosphorylation of neuromodulin at serine 41 disrupts calmodulin binding to the IQ site (Apel et al., 1990; Chapman et al., 1991). A mutational analysis indicated that the mutation S41D also disrupts calmodulin binding. Thus binding of calmodulin to the IQ site of neuromodulin is regulated by the introduction of a negative charge within this region. Significantly, a putative $p34^{cdc2}$ phosphorylation site resides near the IQ sites in Myo2p (Johnston et al., 1991). Alternatively, in a manner analogous to the phosphorylation of myosin light chains, modification of calmodulin rather than Myo2p may regulate the association between calmodulin and Myo2p.

Myo2p, p190, and *dilute* are all implicated in polarized vesicle movement. Immunolocalization studies on p190 and Myo2p and the phenotypes of mutant *myo2* and *dilute* alleles are consistent with a role for class V myosins in vesicle transport (Silvers, 1979; Johnston et al., 1991; Mercer et al., 1991; Espreafico et al., 1992; Lillie, S. H., and S. S. Brown. 1992. *Mol. Biol. Cell.* 3s:42a). Here we demonstrate a specific genetic and biochemical interaction between calmodulin and Myo2p. Future studies will use the genetic techniques available in yeast to define the role of calmodulin, Ca^{2+} and phosphorylation in regulating Myo2p function in polarized growth.

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