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Identification of Functional Connections Between Calmodulin and the Yeast Actin Cytoskeleton

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Manuscript received February 3, 1998
Accepted for publication May 27, 1998

ABSTRACT

One of four intragenic complementing groups of temperature-sensitive yeast calmodulin mutations, *cmd1A*, results in a characteristic functional defect in actin organization. We report here that among the complementing mutations, a representative *cmd1A* mutation (*cmd1-226*: F92A) is synthetically lethal with a mutation in *MYO2* that encodes a class V unconventional myosin with calmodulin-binding domains. Gel overlay assay shows that a mutant calmodulin with the F92A alteration has severely reduced binding affinity to a GST-Myo2p fusion protein. Random replacement and site-directed mutagenesis at position 92 of calmodulin indicate that hydrophobic and aromatic residues are allowed at this position, suggesting an importance of hydrophobic interaction between calmodulin and Myo2p. To analyze other components involved in actin organization through calmodulin, we isolated and characterized mutations that show synthetic lethal interaction with *cmd1-226*; these "*cax*" mutants fell into five complementation groups. Interestingly, all the mutations themselves affect actin organization. Unlike *cax2*, *cax3*, *cax4*, and *cax5* mutations, *cax1* shows allele-specific synthetic lethality with the *cmd1A* allele. *CAX1* is identical to *ANP1*/*GEM3*/*MCD2*, which is involved in protein glycosylation. *CAX4* is identical to the ORF YGR036c, and *CAX5* is identical to *MNN10*/*SLC2*/*BED1*. We discuss possible roles for Cax proteins in the regulation of the actin cytoskeleton.

CALMODULIN, a highly conserved calcium-binding protein, has been implicated in Ca^{2+}-mediated signaling cascades, including muscle contraction and neurotransmitter release (Cohen and Klee 1988). Essential functions of calmodulin for cell proliferation have been studied in diverse eukaryotic cells, including *Saccharomyces cerevisiae* (Davis 1992; Ohya and Anraku 1992), *Schizosaccharomyces pombe* (Takeda and Yamamoto 1987), *Aspergillus nidulans* (Rasmussen *et al.* 1990), and *Aspergillus oryzae* (Yasui *et al.* 1995). It seems likely that calmodulin performs diverse functions by interacting with many different target proteins. Indeed, a large number of calmodulin-binding proteins possessing diverse activities *in vitro* have been identified so far (Cohen and Klee 1988). Many of these proteins have been well characterized biochemically, but it has generally remained unclear which calmodulin targets have functional significance and how these functions are regulated in the cell.

Functions of calmodulin in cell proliferation have been extensively studied in *S. cerevisiae.* The yeast has a single essential calmodulin gene (Davis *et al.* 1986). Because the vertebrate calmodulin is able to functionally replace the endogenous yeast calmodulin (Davis and Thorner 1989; Ohya and Anraku 1989), essential functions of yeast calmodulin can be regarded as the archetype of vertebrates. We previously succeeded in systematic isolation of 14 temperature-sensitive calmodulin mutations by phenylalanine-to-alanine mutagenesis (Ohya and Botstein 1994b). The seven well-conserved phenylalanine residues we mutagenized are likely to be important in interactions with target peptides, as judged by NMR and X-ray structural analysis of the complex between calmodulin and calmodulin-binding peptides (Babu *et al.* 1988; Ikura *et al.* 1991, 1992). The most striking finding from systematic mutagenesis of calmodulin was that the mutations formed four intragenic complementation groups (Ohya and Botstein 1994a). Each group has a characteristic functional defect in actin organization (*cmd1A*), calmodulin localization (*cmd1B*), nuclear division (*cmd1C*), or bud emergence (*cmd1D*). Thus, each complementation group of calmodulin mutations can be thought of as producing a functional defect by loss of interaction with different essential target ligands. Complementing yeast calmodulin mutations have recently proved helpful in dissecting different steps of receptor-mediated endocytosis (Geli *et al.* 1998). Several calmodulin-dependent enzymes, including protein kinases and protein phosphatases, have been studied in yeast. Two essential calmodulin targets

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have thus far been identified in yeast. One is Myo2p (Johnston *et al.* 1991; Brockerhoff *et al.* 1994) and the other is Nuf1p (Geiser *et al.* 1993; Kilmartin *et al.* 1993; Stirling *et al.* 1994). Myo2p is a class V myosin that is involved in polarized growth and functionally implicated in a post-Golgi stage of the secretory pathway (Johnston *et al.* 1991; Govindan *et al.* 1995), although the molecular mechanism is not well understood. Nuf1p plays a role in proper assembly of the spindle pole body, the primary microtubule organizing center in yeast.

To shed more light on the mechanism(s) by which calmodulin affects actin organization, a group of conditional-lethal calmodulin mutants (complementation group A) that displays a defect in intracellular actin organization has been characterized further. A temperature-sensitive calmodulin mutation that falls in this group, *cmd1-226* (F92A), results in loss of localized actin cortical patches in the bud and disappearance of the actin cables at the restrictive temperature (Ohya and Botstein 1994a). Direct binding between calmodulin and actin had not been detected in a gel overlay assay or by sedimenting F-actin in the presence of calmodulin (Piazza and Wallace 1985), suggesting that the phenotypes of *cmd1-226* must be an indirect effect mediated by an as yet unidentified actin-binding protein. Association of calmodulin with actin-binding proteins in other eukaryotes has often been reported. Another example is caldesmon, a calmodulin-binding protein found in smooth muscle that binds F-actin (Sobue *et al.* 1981).

In the budding yeast *S. cerevisiae*, Myo2p appeared to be the only essential calmodulin target known to bind actin. Thus, Myo2p was an obvious candidate to be the mediator of the essential function of calmodulin upon the actin cytoskeleton. Myo2p contains a myosin-like head domain, a series of IQ motifs associated with calmodulin binding, and a C-terminal coiled-coil region, which are well conserved among the members of the class V myosin family (Johnston *et al.* 1991). A temperature-sensitive *myo2-66* mutation encodes a Glu-to-Lys change at position 511, which lies at the actin-binding face in the head domain (Lillie and Brown 1994). The *myo2-66* cells stop growing at the restrictive temperature as large, unbudded cells with mislocalized actin patches (Johnston *et al.* 1991). Immunofluorescence localization of Myo2p at the growing sites of cells further implicates the role of Myo2p in polarized growth (Brockerhoff *et al.* 1994; Lillie and Brown 1994). Physical interaction between Cmd1p and Myo2p was demonstrated both by immunofluorescence microscopy and gel overlay assay (Brockerhoff *et al.* 1994).

We present several lines of genetic and biochemical evidence, suggesting that Myo2p is a downstream target of calmodulin that is essential for actin organization. Analyses of replacement mutations at position 92 of calmodulin, which is altered in *cmd1-226*, indicate that hydrophobic and aromatic interactions are important between Cmd1p and Myo2p. We have also undertaken a genetic screen to identify mutations that show synthetic lethal interaction with *cmd1-226* ("*cax*" mutations). Phenotypic and genetic analyses of *cax* mutants suggest that all the Cax proteins are involved in the regulation of the actin network.

MATERIALS AND METHODS

Yeast strains, media and genetic methods: The strains used in this paper are listed in Table 1. Rich medium (YPD) contains 1% Bacto-yeast Extract (Difco, Detroit, MI), 2% polypeptone (Nihon Seiyaku, Tokyo), and 2% glucose (Wako Chemicals, Osaka, Japan). Synthetic growth medium (SD) for selective growth was described previously, as were methods for tetrad analysis (Kaiser *et al.* 1994). Lithium acetate (Wako Chemicals) was used for yeast transformation with a modification (Schiestl and Gietz 1989) of the original method (Ito *et al.* 1983). FOA agar plates were made by adding 1 μg/ml 5-fluoroorotic acid (Sigma, St. Louis, MO) to SD agar.

DNA manipulations and plasmids: Standard procedures were used for DNA manipulations and *Escherichia coli* transformation (Sambrook *et al.* 1989). Strains DH5αF′ or SCS1 were used to propagate plasmids. DNA sequencing was carried out with an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA) equipped with a fluorescence detector for electrophoretically separated DNA fragments (Connel *et al.* 1987), using both universal primers and primers synthesized specifically as needed. Southern blotting analysis was performed with the ECL gene detection system (Amersham Pharmacia Biotech) to confirm sites of chromosomal integration.

pRB1612 (YCpU-CMD1) containing *CMD1*, *CEN*, and *URA3* was described previously (Ohya and Botstein 1994b).

pRB1612L (YCpL-CMD1) containing a 2-kb *Sal*I-*Bam*HI fragment of *CMD1* was constructed from pRB1612 and pRS315.

pRB1616 (Ohya and Botstein 1994b) was used for subcloning of the *Bst*BI-*Sph*I Phe92 to any amino acid *cmd1* fragment amplified by PCR.

pRB1617 (YIpHade3) (Ohya and Botstein 1994b) was used for chromosomal integration of the Phe92–X *cmd1* mutations. To create YOC376, YOC377, YOC378, YOC379, YOC380, YOC381, and YOC382, an *Sph*I-*Bam*HI fragment containing the Phe92–X *cmd1* mutation from pRB1616-derived plasmid was inserted into pRB1617.

pRB1617L (YIpLade3) was made by replacing the *Bam*HI-*Xho*I *HIS3* fragment of pRB1617 with the 2-kb *Bam*HI-*Sal*I *LEU2* fragment from pJJ282 (Jones and Prakash 1990). To create YOC1170, YOC1172, YOC1174, YOC1175, YOC1176, YOC1178, YOC1180, and YOC1181, the *Sph*I-*Bam*HI fragment containing the allele of the wild-type calmodulin, *cmd1-228*, *cmd1-233*, or *cmd1-239* from the pRB1616-derived plasmid (Ohya and Botstein 1994b), was inserted into pRB1617L.

pYO693 (pYSLU1-CMD1) contains partially defective *CEN3* (Koshland *et al.* 1985), *ADE3*, *URA3*, and *CMD1* (Y. Ohya, unpublished results). The plasmid was used for synthetic lethal screening in the *ade2 ade3* strain.

pYO1148 (YCpL-MYO2) was made by insertion of a 5.5-kb *Cla*I-*Eco*RI fragment of p10-2B (Johnston *et al.* 1991) containing *MYO2* into the *CEN*, *LEU2* plasmid, pRS315 (Sikorski and Hieter 1989).

Random replacement mutagenesis: Four codons at positions 89–92 of *CMD1* were replaced with random sequence using random replacement mutagenesis (Palzkill and Botstein 1992a,b). First, the nucleotide sequence 5′-GCTTTTAAAG TATTC-3′ at positions 88–92 (covering codons 262–276) of *CMD1* was changed to 5′-GCCGGCCTCGAGGGTCTCC-3′ by a two-step PCR method (Ho *et al.* 1989), with the result that the sequence at the region of mutagenesis was replaced with a sequence containing *Nae*I and *Bsa*I recognition sites (un-

TABLE 1

Yeast strains

Strain	Genotype
JP7A[a]	*MAT***a** *ade1 his6 leu2 ura3 myo2-66*
YOC101[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 cmd1-Δ1::TRP1* [pRB1612]
YOC102[b]	*MATα ade2 his3 leu2 lys2 trp1 ura3 cmd1-Δ1::TRP1* [pRB1612]
YOC200[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::CMD1 cmd1-Δ1::TRP1*
YOC226[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1*
YOC228[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-228 cmd1-Δ1::TRP1*
YOC231[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-231 cmd1-Δ1::TRP1*
YOC233[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-233 cmd1-Δ1::TRP1*
YOC239[b]	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-239 cmd1-Δ1::TRP1*
YOC326[b]	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1*
YOC376	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-376 cmd1-Δ1::TRP1*
YOC377	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-377 cmd1-Δ1::TRP1*
YOC378	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-378 cmd1-Δ1::TRP1*
YOC379	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-379 cmd1-Δ1::TRP1*
YOC380	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-380 cmd1-Δ1::TRP1*
YOC381	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-381 cmd1-Δ1::TRP1*
YOC382	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-382 cmd1-Δ1::TRP1*
YOC1100	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::CMD1 cmd1-Δ1::TRP1 myo2-66*[c]
YOC1102	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1 myo2-66*[c]
YOC1105	*MATα ade2 his3 leu2 lys2 trp1 ura3 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1106	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::CMD1 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1107	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1108	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-228 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1109	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-231 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1110	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-233 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1111	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-239 cmd1-Δ1::TRP1 myo2-66*[c] [pRB1612]
YOC1119	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1* [pYO693]
YOC1120	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax1-1* [pYO693]
YOC1126	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax2-1* [pYO693]
YOC1129	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax2-2* [pYO693]
YOC1130	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax3-1* [pYO693]
YOC1132	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax4-1* [pYO693]
YOC1134	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax5-1* [pYO693]
YOC1135	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-226 cmd1-Δ1::TRP1 cax5-1* [pYO693]
YOC1139	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1 cax1-1*
YOC1140	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1 cax2-2*
YOC1141	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1 cax3-1*
YOC1142	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1 cax4-1*
YOC1143	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1 cax5-1*
YOC1170	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1*
YOC1172	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::cmd1-228 cmd1-Δ1::TRP1*
YOC1174	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::cmd1-233 cmd1-Δ1::TRP1*
YOC1175	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::cmd1-239 cmd1-Δ1::TRP1*
YOC1176	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::CMD1 cmd1-Δ1::TRP1*
YOC1178	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::cmd1-228 cmd1-Δ1::TRP1*
YOC1180	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::cmd1-233 cmd1-Δ1::TRP1*
YOC1181	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::LEU2::cmd1-239 cmd1-Δ1::TRP1*
YOC2288	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-228 cmd1-Δ1::TRP1 myo2-66*[c]
YOC2289	*MATα ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-231 cmd1-Δ1::TRP1 myo2-66*[c]
YOC2290	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-233 cmd1-Δ1::TRP1 myo2-66*[c]
YOC2291	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3::cmd1-239 cmd1-Δ1::TRP1 myo2-66*[c]

[a] Johnston *et al.* (1991).
[b] The strains were described previously (Ohya and Botstein 1994b).
[c] The *myo2-66* allele in a strain JP7A was crossed into our background.

derlined). Second, this new oligonucleotide sequence was released by digestion with *Nae*I and *Bsa*I, which created a 12-bp deletion. To replace the deleted nucleotides with random sequence, a second linker (made from oligonucleotides 5′-NNNNNNGGAGACCGTCGACTTGTGAGCGGATAACAGGT CTCG-3′ and 5′-NNNNNNCGAGACCTGTTATCCGCTCACAA GTCGACGGTCTCC-3′ by annealing following blunting) was inserted: it contains 6 bp of random sequence at each end,

TABLE 2

Primers used for PCR-based mutagenesis

Primer	Oligonucleotide sequence
CMD1N+	ATG TCT TCG AAT CTT ACC GAA GAA CAA ATT
CMD1C−	GGC CCG CAT GCC TTG GTA AAC AAT CCG TAT
CMD1(F92V)+	AA CTA CTA GAA GCT TTT AAA GTA GTC GAT AAG AAC GGT G
CMD1(F92V)−	C ACC GTT CTT ATC GAC TAC TTT AAA AGC TC TAG TAG TT
CMD1(F92I)+	AA CTA CTA GAA GCT TTT AAA GTA ATC GAT AAG AAC GGT G
CMD1(F92I)−	C ACC GTT CTT ATC GAT TAC TTT AAA AGC TC TAG TAG TT
CMD1(F92L)+	AA CTA CTA GAA GCT TTT AAA GTA CTC GAT AAG AAC GGT G
CMD1(F92L)−	C ACC GTT CTT ATC GAG TAC TTT AAA AGC TC TAG TAG TT
CMD1(F92W)+	AA CTA CTA GAA GCT TTT AAA GTA TGG GAT AAG AAC GGT G
CMD1(F92W)−	C ACC GTT CTT ATC CCA TAC TTT AAA AGC TC TAG TAG TT
CMD1(F92M)+	AA CTA CTA GAA GCT TTT AAA GTA ATG GAT AAG AAC GGT G
CMD1(F92M)−	C ACC GTT CTT ATC CAT TAC TTT AAA AGC TC TAG TAG TT
CMD1(F92C)+	AA CTA CTA GAA GCT TTT AAA GTA TGC GAT AAG AAC GGT G
CMD1(F92C)−	C ACC GTT CTT ATC GCA TAC TTT AAA AGC TC TAG TAG TT
CMD1(F92Y)+	AA CTA CTA GAA GCT TTT AAA GTA TAC GAT AAG AAC GGT G
CMD1(F92Y)−	C ACC GTT CTT ATC GTA TAC TTT AAA AGC TC TAG TAG TT

+ and −, sense and antisense strands, respectively. Nucleotide changes are underlined.

along with embedded *Bsa*I recognition sites. Libraries of independent linker insertions were constructed in *E. coli*, and the plasmid DNA was extracted and purified. The DNA was digested with *Bsa*I again and religated, leaving an insertion of 12 random nucleotides.

Three independent libraries containing *CMD1* random substitution mutations (*i.e.*, altered sequences still capable of producing functional calmodulin) at positions 89–92 were used in this study. Plasmids containing these *CMD1* mutations were recovered from yeast, amplified in *E. coli*, and transformed back into yeast to ensure that the phenotype was conferred by the plasmid rather than by any spontaneous genomic mutations. DNA sequencing of random replacement at positions 89–92 revealed that every plasmid had a different amino acid sequence.

PCR-based mutagenesis: Introduction of mutations at position 92 was performed with a two-step PCR-based mutagenesis (Ho *et al.* 1989), as described before (Ohya and Botstein 1994b), using 16 oligonucleotides (Table 2). CMD1N+, corresponding to the translation start sequence of calmodulin, contained an artificial *Bst*BI site without changing any amino acid sequence, and CMD1C−, corresponding to the 3′-noncoding sequence, contained the *Sph*I site at the end. Long primers with Phe to Val, Ile, Leu, Trp, Met, Cys, and Tyr mutations were made in both sense and antisense strands.

In the first PCR reaction, both N- and C-terminal fragments were amplified separately and purified after electrophoresis on an agarose gel. These two fragments shared an overlapping region of at least 18 bp containing the mutations at position 92, so that in a second PCR reaction both fragments were mixed and the whole fragment was amplified with CMD1N+ and CMD1C− primers. The fragment containing the mutation was digested with *Bst*BI and *Sph*I, and was subcloned into the *Bst*BI-*Sph*I gap of pRB1616. All mutations were verified by DNA sequencing. DNA sequencing with two sequencing primers (5′-TGACCGGAAACTACTGAAC-3′ and 5′-GATGA ACGAAATAGATGTTGATGG-3′) sufficed to cover the entire coding sequence of calmodulin.

TABLE 3

***cmd1* alleles used in this study**

Allele	Mutation site
cmd1-226[a]	F92A
cmd1-228[a]	F12A F16A F19A
cmd1-231[a]	F12A F89A
cmd1-233[a]	F12A F140A
cmd1-239[a]	F65A F68A
cmd1-376	F92V
cmd1-377	F92I
cmd1-378	F92L
cmd1-379	F92W
cmd1-380	F92M
cmd1-381	F92C
cmd1-382	F92Y

[a]*cmd1* alleles described previously (Ohya and Botstein 1994b).

Construction of yeast strains: To integrate *cmd1* mutations into the genome, we used the pRB1617-derived plasmids (using *HIS3* as a selectable marker) or pRB1617L-derived plasmids (using *LEU2* as a selectable marker). After digestion of these plasmids with *Sac*II and *Alw*NI, the *ade3-cmd1-HIS3-ade3* and *ade3-cmd1-LEU2-ade3* fragments were used to transform YOC101 (*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 cmd1-Δ1::TRP1* [pRB1612]) or YOC102 (same as YOC101 except its mating type). Correct integrants were recognized as white transformants (*ade2 ade3*). Finally, strains that had lost pRB1612 were selected on FOA plates. The resulting calmodulin mutant strains are listed in Table 1. The *cmd1* mutations used in this study are listed in Table 3.

To construct *cmd1 myo2-66* double mutants carrying the wild-type calmodulin on a *URA3* plasmid, crosses were made between YOC1105 (*MAT*α *ade2 his3 leu2 lys2 trp1 ura3 cmd1-Δ1::TRP1 myo2-66* [pRB1612]) and representative calmodulin mutants (*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 ade3::HIS3:: cmd1-xxx cmd1-Δ1::TRP1*). The calmodulin strains used were YOC200, YOC226, YOC228, YOC231, YOC233, YOC239, YOC376, YOC377, YOC378, YOC379, YOC380, YOC381, and YOC382. After mating, the resulting diploids were sporulated. Segregants from the tetrads that simultaneously showed His^+,

Ura$^+$, and Ts phenotypes were taken to be *myo2-66 cmd1* double mutants harboring the wild-type calmodulin gene on a *URA3* plasmid.

Purification and biotin labeling of calmodulins: *E. coli* strains carrying the appropriate plasmids were incubated for 2 hr at 37° in TB medium containing 100 μg/ml ampicillin. Calmodulin production was induced by adding isopropyl l-β-d-thiogalactopyranoside to a final concentration of 1 mm. After a 2-hr incubation, cells were collected by centrifugation, washed twice in 50 mm Tris-HCl, pH 7.5, containing 1 mm phenylmethylsulfonyl chloride and 1 mm $MgCl_2$, and freeze thawed. Cellular debris was removed by ultracentrifugation for 30 min at 200,000 × *g*. Calmodulin was purified essentially as described (Ohya *et al.* 1987; Takahashi *et al.* 1996). The 2-ml supernatant was applied to the phenyl-sepharose column chromatography, and then the calmodulin was eluted with 50 mm Tris-HCl, pH 7.5, containing 5 mm EGTA and 0.1 m ammonium sulfate. Purified wild-type and mutant calmodulins were biotin labeled using ImmunoPure Sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL).

Calmodulin-Myo2-binding assay: The procedures were based on those of Brockerhoff *et al.* (1994).

Myo2 fusion protein was made using the GST Gene Fusion System (Pharmacia, Piscataway, NJ). The fragment of *MYO2* that encodes residues 908–1086 of Myo2p was cloned into the pGEX plasmid. Myo2 fusion protein was expressed in an *E. coli* strain. Proteins (50 μg) were separated on a 10% SDS polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane. The transfer buffer was 48 mm Tris and 39 mm glycine. After transfer, the membrane was blocked for 1 hr in blocking buffer (50 mm Tris-HCl, 150 mm NaCl, 0.05% Tween 20, 2% nonfat dry milk) and then probed at room temperature for 1.5 hr with 2.4 μm biotin-labeled wild-type or mutant calmodulin in Ca^{2+} buffer (10 mm Tris, 0.2% BSA, 1 mm $CaCl_2$) or EGTA buffer (10 mm Tris, 0.2% BSA, 10 mm EGTA). The membrane was then washed three times each for 10 min and air dried. Biotinylated horseradish peroxidase–avidin complex was formed using the Vectastain Elite standard kit (Vector Laboratories, Burlingame, CA) and stained using the POD immunostain set (Wako Chemicals).

Immunofluorescence microscopy: Procedures for phalloidin staining and immunofluorescence microscopy were based on those of Pringle *et al.* (1989). Actin staining was carried out using rhodamine-conjugated phalloidin, as a 3.3 μm stock in methanol from Molecular Probes (Eugene, OR). For staining of Myo2p, rabbit anti-Myo2p antibody (Lillie and Brown 1994) was used as a first antibody, biotinylated anti-rabbit antibody as a second antibody, and fluorophore-conjugated avidin, streptoavidin (Boehringer Mannheim, GmbH, Germany). For staining of Cmd1p, rabbit anti-Cmd1p antibody (Ohya *et al.* 1987) was used as a first antibody, and FITC-labeled anti-rabbit antibody was used as a second antibody. Stained cells were examined with an Olympus BX-FLA epifluorescence microscope (Olympus, Tokyo) and photographed on T-MAX 400 film (Eastman Kodak Co., Rochester, NY).

Isolation of *cax* mutants: A colony-sectoring assay was used to screen mutations that create a requirement for *CMD1* in *cmd1-226* cells. The *ade2 ade3 cmd1-226* strain carrying the wild-type calmodulin gene on a pYSLU1-CMD1 (YOC1119) was used as a parent strain. pYSLU1-CMD1 contains a mutation in the *CEN3* region that makes the plasmid unstable. The principle of the colony-sectoring assay is that *ade2* strains form red colonies, that *ade2 ade3* strains form white colonies, while *ade2 ade3* mutants with an *ADE3* plasmid often lose the plasmid and produce abundant white sectors in an otherwise red colony. Therefore, after mutagenesis of YOC1119, one can get the mutants that require a calmodulin by screening for nonsectoring colonies. YOC1119 was mutagenized with EMS to ~70% survival (Sherman *et al.* 1974). The mutagenized cells were plated on YPD plates and incubated at 23° for 7 days. Red colonies were picked and rechecked for nonsectoring phenotype and for plasmid loss phenotype on plates containing 1 μg/ml FOA. The dependency of the strains on *CMD1* was tested by transformation of a second copy of *CMD1* on the *LEU2/CEN* plasmid (YCpL-CMD1) into the mutants. Only mutants whose sectoring phenotype and FOA sensitivity were restored by the second copy of *CMD1* were analyzed further. Tetrad analysis was then carried out to pick up strains whose FOA sensitivity and nonsectoring phenotype were caused by a single mutation. Complementation tests were performed by mating mutant segregants and testing FOA sensitivity and sectoring phenotype on YPD plates.

Construction of *cax* mutants with an integrated *CMD1* background: The *cax* strains with an integrated *CMD1* background were constructed by crossing the *cax* strains originally isolated by colony-sectoring assay (*cax ade3::cmd1-226::HIS3* [pYSLU1-CMD1]) with the wild-type strain YOC1170 (*MAT***a** *CAX ade3::CMD1::LEU2*) or YOC1176 (same as YOC1170 except its mating type). The resulting diploids were sporulated. To isolate the *cax CMD1::LEU2* strains, sensitivity to FOA, *LEU*, and *HIS* markers were checked. If two His$^+$ segregants (with *ade3::cmd1-226::HIS3*) of a tetrad showed an FOA-insensitive phenotype, then the two other Leu$^+$ segregants were assigned to harbor *cax* mutations. The possibility of gene conversion at the *CAX* locus was discarded by confirming that the phenotypes of the two possible *cax* segregants were the same.

Cell lysis assay: Yeast cells were incubated on a YPD plate at 23° for 3 days. The plate was then overlaid with an alkaline phosphatase assay solution as described by Paravicini *et al.* (1992). Colonies containing lysed cells turned blue within 1 hr, while control colonies remained unstained, even after 2 hr.

Cloning and genetic mapping of *CAX1*: *CAX1* was cloned by complementation of the FOA-sensitive growth phenotype of the corresponding mutant, using a YEp-based genomic library made by Yoshihisa and Anraku (1989). Plasmids that yielded FOA-insensitive transformants of the strain YOC1120 (*cmd1-226::HIS3 cax1-1* [pYSLU1-CMD1]) were recovered. The plasmids were then checked to see whether they contained a *CMD1* gene, both by restriction mapping and by PCR with primers that amplified the ORF of *CMD1*. Two plasmids that conferred FOA-insensitive phenotype in *cax1* cells were recovered. One turned out to contain *CMD1*, and the other plasmid, pMS102, was studied further.

One end of the *cax1*-rescuing fragment was sequenced using the primer suited for direct sequencing of the DNA insert of YEp13. Comparison with the sequence database revealed that the insert was within a region of chromosome V. Subcloning analyses indicated that the 3-kb *Sal*I-*Spe*I fragment has complementation activity on a single-copy plasmid and that *Nco*I is an essential site. The open reading frame defined by these tests is YEL036C, which encodes a previously characterized gene, *ANP1* (also known as *GEM3* and *MCD2*).

To show that the plasmid that confers the FOA-insensitive phenotype actually contains the *CAX1* gene, integration mapping was carried out. The *Eco*RV-*Spe*I fragment located ~300 bp downstream from the *ANP1* ORF was replaced with a *LEU2* fragment from pJJ283 in YOC326 (*MAT*α *cmd1-226::HIS3 leu2*). The integrant was mated to YOC1120. Tetrad analysis revealed that the *LEU2* marker was tightly linked to the *CAX1* locus (31 tetrads analyzed). Thus, we concluded that pMS102 contained the *CAX1* gene itself.

RESULTS

The *myo2-66* mutation shows synthetic lethal interaction with *cmd1-226*: The yeast calmodulin mutant *cmd1-*

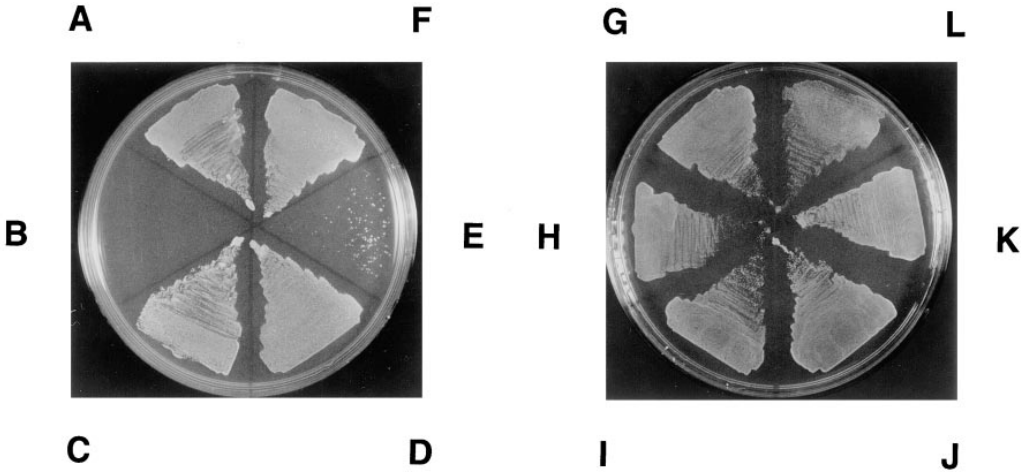

Figure 1.—Figure 1. — *cmd1-226* shows synthetic lethal interaction with *myo2-66*. Cells of *cmd1 myo2-66* double mutants carrying the wild-type calmodulin gene on a *URA3* plasmid were fully grown in YPD from single colonies at 23°. The cell suspensions were diluted with 1 μg/ml FOA medium and incubated at 23° for 2 days to allow Ura$^-$ cells to grow. The equal volumes of the liquid culture were streaked on an FOA-containing plate (A–F) or on a synthetic complete plate (G–L). The following strains were tested: (A and G) YOC1106 (*CMD1 myo2-66* [YCpU-CMD1]), (B and H) YOC1107 (*cmd1-226 myo2-66* [YCpU-CMD1]), (C and I) YOC1108 (*cmd1-228 myo2-66* [YCpU-CMD1]), (D and J) YOC1109 (*cmd1-231 myo2-66* [YCpU-CMD1]), (E and K) YOC1110 (*cmd1-233 myo2-66* [YCpU-CMD1]), (F and L) YOC1111 (*cmd1-239 myo2-66* [YCpU-CMD1]).

226 has a characteristic functional defect in actin organization (Ohya and Botstein 1994a). At the permissive temperature, the temperature-sensitive *cmd1-226* cells grow slower than wild-type cells and frequently contain delocalized actin. At the restrictive temperature, >95% cells have lost localized actin cortical patches in the bud. In an attempt to genetically identify the essential calmodulin target that functions in the organization of the actin network, we looked for synthetic lethal interaction between the *cmd1-226* mutation and a mutation in the *MYO2* gene that encodes the only essential target of calmodulin known to bind actin. In the *myo2-66* mutant, aberrant actin morphology had been observed, suggesting that Myo2p plays some role in actin organization (Johnston *et al.* 1991).

By tetrad dissection, we constructed several *cmd1 myo2-66* double-mutant strains harboring the wild-type *CMD1* on a *URA3*-marked plasmid. The calmodulin alleles used were *cmd1-226* (F92A) with a defect in actin organization, *cmd1-228* (F12A F16A F19A) with a defect in calmodulin localization at sites of cell surface growth, *cmd1-231* (F12A F89A) with a defect in bud emergence, *cmd1-233* (F12A F140A) with a defect in bud emergence, and *cmd1-239* (F65A F68A) with a defect in nuclear division. The double-mutant strains harboring the *URA3-CMD1* plasmid were then tested for sensitivity to FOA. Only strains that can lose the *URA3-CMD1* plasmid are able to grow on FOA medium.

We found that *myo2-66* is synthetically lethal with *cmd1-226*. The *cmd1-226 myo2-66* double mutant failed to grow at 23°, although each of the single mutants grew at this temperature (Figure 1B). The strains with either a *cmd1-228 myo2-66*, *cmd1-231 myo2-66*, or *cmd1-239 myo2-66* mutation grew well on FOA medium at 23° (Figure 1, C, D, and F). Instead, the *cmd1-233 myo2-66* double mutant grew slowly, but eventually formed colonies (Figure 1E). We randomly picked 10–15 colonies of *cmd1-233 myo2-66* from FOA medium and confirmed that they all had lost the *URA3-CMD1* plasmid, indicating that the *cmd1-233 myo2-66* double mutant is viable. We examined the doubling time and restrictive temperature of the *cmd1 myo2-66* double mutants (Table 4). Among the viable double mutants, *cmd1-233 myo2-66* exhibited the slowest growth rate. However, the restrictive temperature of the *cmd1-233 myo2-66* mutant was the same as that of *myo2-66*. The other double mutants showed no obvious synthetic effect on doubling time or on restrictive temperature. Thus, a synthetic growth defect of *cmd1-233 myo2-66* was observed, but it was not as strong as that of *cmd1-226 myo2-66*.

Cmd1-226p has decreased binding activity to Myo2p: To examine directly the possible impairment of the ability of the mutant calmodulin to bind Myo2p, we analyzed the binding of a Myo2p fusion protein with wild-type and several mutant calmodulins by a gel overlay assay. The wild-type and mutant calmodulins used in this experiment exhibited a single band after running on an SDS-PAGE gel (Figure 2A). A DNA segment of *MYO2* containing the coding sequence of the sixth IQ motif was fused to the glutathione *S*-transferase gene to produce a Myo2p fusion in *E. coli* (Figure 2B, lane 2). Figure 2B compares the binding ability of each mutant calmodulin with that of the wild-type calmodulin in the presence of Ca^{2+}. Binding of Cmd1-226p to the Myo2p fusion was severely decreased. We detected a definite binding signal of Cmd1-228p, Cmd1-231p, Cmd1-233p, and Cmd1-239p to Myo2p fusion protein, although the binding of some mutant calmodulins may be slightly

TABLE 4

Doubling time and restrictive temperature of *cmd1*, *myo2*, and *cmd1 myo2* mutants

Genotype	Doubling time at 23° (hr)	Restrictive temperature
Wild type	3.2	
myo2-66	3.2	30°
cmd1-226	3.9	37°
cmd1-228	3.2	35.5°
cmd1-231	3.8	37.5°
cmd1-233	3.6	37°
cmd1-239	3.4	37°
cmd1-226 myo2-66	[a]	[a]
cmd1-228 myo2-66	4.0	30°
cmd1-231 myo2-66	3.7	30°
cmd1-233 myo2-66	6.7	30°
cmd1-239 myo2-66	4.1	30°

[a] Doubling time and restrictive temperature of the *cmd1-226 myo2* strain cannot be determined because it was not viable.

Doubling times [expressed as time (hours) required to double turbidity at 600 nm] of the strains were measured in rich (YPD) medium at 23° using two independent colonies. Experimental errors were <10%. The strains were tested for growth on YPD plates at every 1° between 28° and 35° and at every 0.5° between 35° and 37.5° for 3 days, except for the wild-type strain. The restrictive temperatures are indicated. The strains used are as follows: YOC200 (wild type), YOC1100 (*myo2-66*), YOC226 (*cmd1-226*), YOC228 (*cmd1-228*), YOC231 (*cmd1-231*), YOC233 (*cmd1-233*), YOC239 (*cmd1-239*), YOC2288 (*cmd1-228 myo2-66*), YOC2289 (*cmd1-231 myo2-66*), YOC2290 (*cmd1-233 myo2-66*), and YOC2291 (*cmd1-239 myo2-66*).

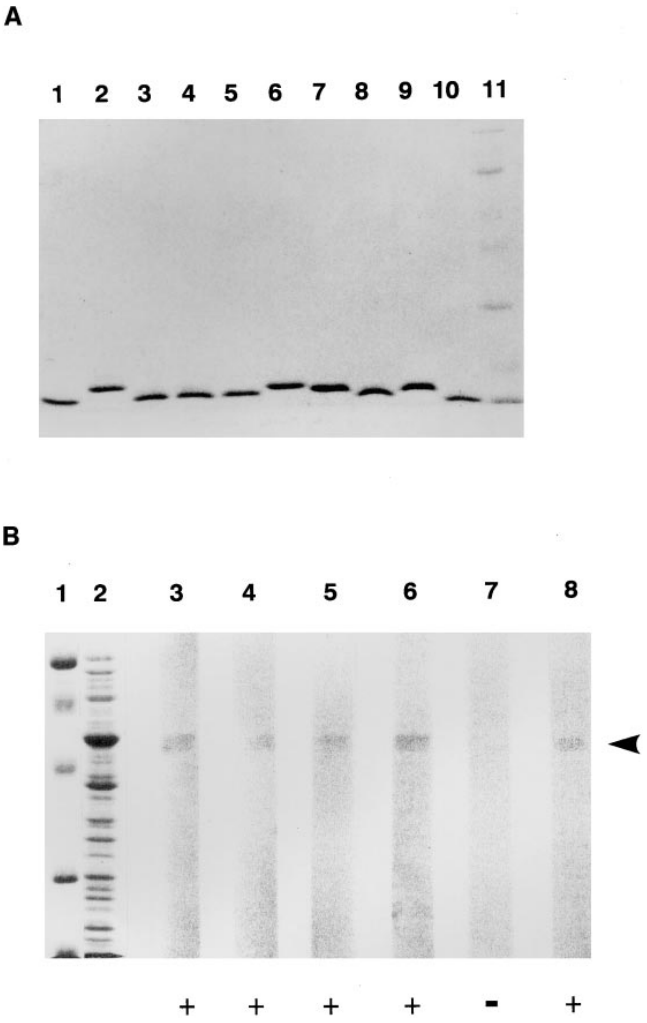

Figure 2.—Purification of calmodulins and Cmd1p-Myo2p-binding assay. (A) Purification of wild-type and mutant calmodulins. Purified wild-type and various mutant calmodulins were subjected to SDS-PAGE. Lane 1, Cmd1-226p; lane 2, Cmd1-228p; lane 3, Cmd1-231p; lane 4, Cmd1-232p; lane 5, Cmd1-233p; lane 6, Cmd1-234p; lane 7, Cmd1-239p; lane 8, Cmd1-240p; lane 9, Cmd1-250p; lane 10, wild-type calmodulin; lane 11, markers (mol. wt. = 200, 97.4, 69, 46, 30, 21.5, and 14.3 kD). (B) Binding assay of Myo2p and each mutant calmodulin. Lane 1, markers (mol. wt. = 97.4, 69, 46, 30, and 21.5 kD). Myo2-GST fusion protein was expressed and subjected to SDS-PAGE gel (lane 2). The fusion was blotted with wild-type calmodulin (lane 3), Cmd1-228p (lane 4), Cmd1-239p (lane 5), Cmd1-231p (lane 6), Cmd1-226p (lane 7), or Cmd1-233p (lane 8). An arrowhead indicates the band of the Myo2-GST fusion protein. Binding results are expressed as the density of bands: +, binding; −, decreased binding. The experiment was performed more than twice, and we obtained the same results.

weaker compared with that of a wild-type calmodulin. When we performed the same experiment in the absence of Ca^{2+} (using 10 mm EGTA buffer), no binding was observed for each calmodulin (data not shown). It was reported that binding of calmodulin to the last four IQ motifs is Ca^{2+} independent, while binding to the first two IQ motifs was inhibited by Ca^{2+} (Brockerhoff *et al.* 1994). We used the last (the sixth) IQ motif in this study, and Ca^{2+} seems to have different effects on the interactions of the different last four IQ motifs with calmodulin.

The result indicated that the *cmd1-226* mutation results in a severe defect in binding to the Myo2p fusion protein. Because the *cmd1-226* mutation contains only the phenylalanine-to-alanine change at position 92, Phe92 is likely to be important for the proper interaction with the IQ motif of Myo2p. Together with the genetic result of synthetic lethality between *cmd1-226* and *myo2-66*, we suggest that decreased binding of Cmd1-226p to Myo2p, in combination with the weak interaction between Myo2p and actin, results in lethality. Because Cmd1-233p showed binding to Myo2p, our interpretation is that *cmd1-233* does not mainly affect Myo2p function (see discussion).

The *cmd1-226* mutation does not affect Myo2p localization, but affects Cmd1p localization: We immunolocalized Myo2p and Cmd1p in the *cmd1-226* cells. As reported previously (Brockerhoff *et al.* 1994; Lillie and Brown 1994), a Myo2p cap was seen in small and unbudded cells of the wild-type strain (Figure 3A). We found that apparent Myo2p staining remained at the bud tip in >90% of the *cmd1-226* cells, and that the

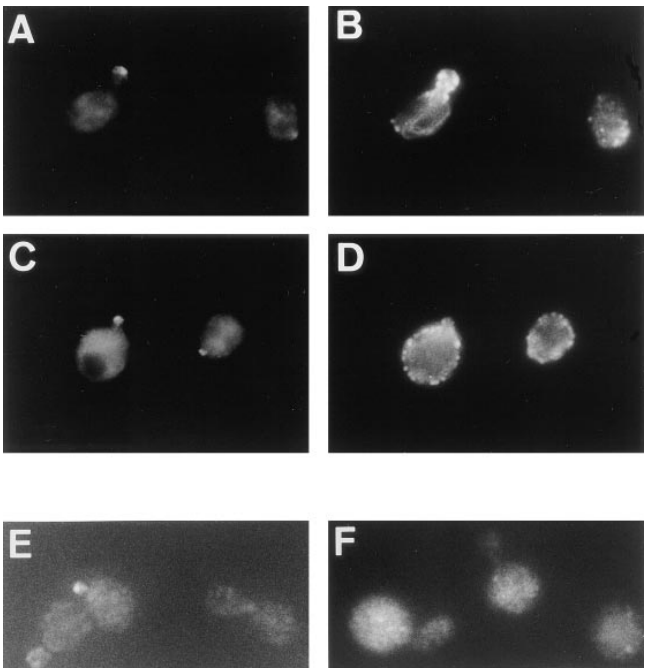

Figure 3.—Myo2p and Cmd1p localization in wild-type and *cmd1-226* cells. Cells were shifted from 27° to 33° for 170 min. The cells were then double labeled with anti-Myo2 antibody (A and C) and rhodamine phalloidin (B and D), or cells were labeled with anticalmodulin antibody (E and F). Strains: (A, B, and E) YOC200 (wild type) and (C, D, and F) YOC226 (*cmd1-226*).

staining was seen clearly in cells that had lost actin spot polarization (Figure 3, C and D).

In the *cmd1-226* cells, polarized calmodulin localization was lost (Figure 3F). The loss of calmodulin localization is a property shared between *cmd1-226* (*cmd1A*) and *cmd1-228* (*cmd1B*). Under the same conditions, however, *cmd1-226* has delocalized actin organization, while *cmd1-228* has wild-type actin organization (Ohya and Botstein 1994a). Because *cmd1A* and *cmd1B* complement each other, it seems likely that the primary defects of these mutants are different. Based on the fact that localization of calmodulin is dependent on actin (Brockerhoff and Davis 1992), our current hypothesis is that the loss of calmodulin localization in *cmd1A* cells is caused by the indirect effect of delocalized actin organization.

Random replacement mutagenesis of calmodulin at positions 89–92: The phenylalanine-to-alanine alteration at position 92 impairs the essential function of calmodulin. To analyze the requirement of amino acid residues at position 92 and to compare requirements at different phenylalanine residues, we analyzed >50 calmodulin mutations replaced with random sequences at positions 89–92 (see materials and methods). This region contains two phenylalanine residues (Phe89 and Phe92). Table 5 shows the properties of the replacement mutants, including growth phenotypes and the predicted amino acid residues. We found a strong preference at position 92. Phenylalanine, valine, isoleucine, and leucine at position 92 were recovered in the sequence, resulting in robust growth at any temperature. Alanine and tryptophan were recovered only in the temperature-sensitive mutations. No charged amino acid was allowed (the probability that this occurs by chance is $P < 7 \times 10^{-4}$). Amino acids with neither the hydroxyl nor amide group were allowed (the probability that this occurs by chance is $P < 7 \times 10^{-4}$). These results suggested that hydrophobic and aromatic amino acid residues are the only ones consistent with function at position 92. In contrast, we found no strong preference at position 89. Charged and polar amino acids were allowed at this position, although they appeared only in "Ts" and "partial Ts" mutants (Table 5).

Site-directed mutagenesis at the position Phe92: Random replacement mutagenesis uses a functional selection approach suited for determining which are the most critical amino acid residues in the randomizing region. This technique, however, does not always give a perfect solution for the amino acid requirement, partly because only a statistical interpretation is available for the amino acids that are not recovered in the complementing sequence, and partly because the individual replacements usually contain additional amino acid changes. To obtain more accurate information for the requirement at position 92, we used site-directed mutagenesis at this position.

We constructed seven replacement mutations at position 92, resulting in an alteration to valine, isoleucine, leucine, tryptophan, methionine, cysteine, or tyrosine, based on the results of random replacement mutagenesis. Valine, isoleucine, and leucine were expected to be "good" residues at position 92. Tryptophan was expected to be an "allowed but temperature-sensitive" residue. Not even statistical data were available for methionine, cysteine, and tyrosine. All the mutants were constructed by insertion of the calmodulin mutations into the genome after verifying the mutations. We found that all of the seven mutants constructed grew at 38°, as well as at 25° (Table 6). The phenotype of the strain with the tryptophan replacement mutation (*cmd1-379*) was particularly unexpected because all the random replacement mutants with Trp at position 92 exhibited a temperature-sensitive phenotype. On the basis of these results, we assume that additional mutations at positions 89–91 in *cmd1-307*, *cmd1-308*, *cmd1-309*, *cmd1-310*, *cmd1-325*, and *cmd1-326* (Table 5) were involved in the temperature-sensitive phenotypes observed for these random replacement mutants.

The actin morphology of the site-directed mutants was examined for comparison with that of *cmd1-226* (F92A). The mutant cells were shifted from 27° to 33°, incubated for 170 min, and then stained with rhodamine-phalloidin. We found that none of the mutants, other than *cmd1-226* (F92A) cells, have defects in actin

TABLE 5

Results of random replacement mutagenesis of calmodulin at positions 89–92

Allele	Amino acid 89	90	91	92	Growth
Wild type	Phe*	Lys*	Val*	Phe*	Wild type
cmd1-274	Ala*	Met*	Cys*	Ile*	Wild type
cmd1-275	Leu*	Ser*	Ile*	Leu*	Wild type
cmd1-276	Ile*	Arg*	Ile*	Leu*	Wild type
cmd1-277	Trp*	Leu*	Thr*	Leu*	Wild type
cmd1-278	Phe*	Val*	Leu*	Leu*	Wild type
cmd1-279	Val*	Lys*	Thr*	Phe*	Wild type
cmd1-280	Val*	His*	Leu*	Phe*	Wild type
cmd1-281	Phe*	Ser*	Cys*	Val*	Wild type
cmd1-282	Leu*	Ala*	Ile*	Val*	Wild type
cmd1-283	Tyr†	His*	Leu*	Ala†	Ts
cmd1-284	Trp*	Ala*	Ile*	Ala†	Ts
cmd1-285	Trp*	Pro	Leu*	Ala†	Ts
cmd1-286	Tyr†	Phe	Leu*	Cys	Ts
cmd1-287	Trp*	Tyr	Phe	Cys	Ts
cmd1-288	Asn†	Arg*	Leu*	Ile*	Ts
cmd1-289	Tyr†	Ala*	Gln†	Ile*	Ts
cmd1-290	His†	Tyr	Leu*	Leu*	Ts
cmd1-291	Tyr†	Arg*	His	Leu*	Ts
cmd1-292	Asn†	Gln	Leu*	Leu*	Ts
cmd1-293	His†	Arg*	Thr*	Leu*	Ts
cmd1-294	His†	His*	Cys*	Leu*	Ts
cmd1-295	Leu*	Ala*	Gln†	Leu*	Ts
cmd1-296	Val*	Ala*	Leu*	Leu*	Ts
cmd1-297	His†	Arg*	Leu*	Leu*	Ts
cmd1-298	Gln	Cys	Ile*	Leu*	Ts
cmd1-299	His†	Tyr	Leu*	Leu*	Ts
cmd1-300	Asn†	Ser*	Leu*	Leu*	Ts
cmd1-301	Gly†	Ser*	Leu*	Leu*	Ts
cmd1-302	Trp*	Arg*	Met	Met	Ts
cmd1-303	Gln	Arg*	Val*	Met	Ts
cmd1-304	Arg†	Arg*	Thr*	Phe*	Ts
cmd1-305	His†	Leu*	Leu*	Phe*	Ts
cmd1-306	Arg†	Arg*	Leu*	Phe*	Ts
cmd1-307	His†	Arg*	Ala	Trp†	Ts
cmd1-308	Leu*	Thr	Ser	Trp†	Ts
cmd1-309	Ile*	Val*	Leu*	Trp†	Ts
cmd1-310	Met	Asn	His	Trp†	Ts
cmd1-311	His†	Arg*	Leu*	Tyr	Ts
cmd1-312	Tyr†	Arg*	Ile*	Val*	Ts
cmd1-313	His†	Asn	Leu*	Val*	Ts
cmd1-314	Leu*	Arg*	Trp†	Val*	Ts
cmd1-315	Phe*	Gln	Lys†	Ile*	Partial Ts
cmd1-316	Phe*	Phe†	Thr*	Ile*	Partial Ts
cmd1-317	Thr†	Arg*	Leu*	Ile*	Partial Ts
cmd1-318	Phe*	Leu*	Lys†	Leu*	Partial Ts
cmd1-319	Gly†	Ser*	Leu*	Leu*	Partial Ts
cmd1-320	Ser†	Ala*	Leu*	Leu*	Partial Ts
cmd1-321	Phe*	Leu*	Lys†	Leu*	Partial Ts
cmd1-322	Leu*	Gly	Phe	Met	Partial Ts
cmd1-323	Gln	Arg*	Phe	Phe*	Partial Ts
cmd1-324	Tyr†	Ser*	Leu*	Phe*	Partial Ts
cmd1-325	Val*	Ser*	Phe	Trp†	Partial Ts
cmd1-326	Val*	Leu*	Cys*	Trp†	Partial Ts

Growth of the *cmd1* strains was examined on YPD at 37°. Growth at 37° is indicated as follows: Wild type, growth equivalent to wild type; Ts, no growth; partial Ts, poor growth. Amino acids that appeared in wild-type sequences are marked with an asterisk. If three amino acids are marked with an asterisk in the Ts or partial Ts mutants, the rest is expected to cause Ts phenotype and marked with a dagger. When only one or two amino acid(s) are marked with an asterisk, we cannot tell which of the rest causes Ts phenotype.

organization. While <10% small-budded *cmd1-226* cells contained a polarized actin network (Table 6; Figure 4B), >85% small-budded cells of other site-directed mutants had polarized actin organization (Table 6; Figure 4, C–E).

Of eight replacement mutations, *cmd1-226* (F92A) and *cmd1-380* (F92M) mutations showed synthetic lethal interaction with *myo2-66* (Table 6). However, the F92M calmodulin can bind to the Myo2p fusion and to wild-type calmodulin, based on the gel overlay assay (data not shown).

Isolation of mutations that show synthetic lethal interaction with *cmd1-226*: We sought to identify additional components involved in the regulation of actin organization by calmodulin by screening for synthetic lethal interactions with *cmd1-226*, as found for *myo2-66*. As described in materials and methods, we began with a *cmd1-226* also carrying an intact plasmid-borne *CMD1* gene, and we isolated eight recessive mutations that display recessive lethality with *cmd1-226*. We called this phenotype Cax (calmodulin-dependent in cmd one two twenty-six). The *cax* mutants harboring the wild-type *CMD1* gene on a *URA3*-marked plasmid failed to grow on FOA plates (Figure 5A); using this phenotype, we carried out complementation analysis, which revealed that the *cax* mutations could be divided into five complementation groups. All the mutants became able to grow on FOA plates after transformation with the second plasmid carrying the wild-type *CMD1* gene (Figure 5B).

Allelism of each *cax* group was tested with *myo2-66* because *myo2-66* also showed synthetic lethal interaction with *cmd1-226*. Cax strains were transformed with a single-copy plasmid pYO1148 containing *MYO2*. The plasmid failed to complement the FOA sensitivity of the *cax* mutations, indicating that none of the recessive *cax* mutations are allelic to *myo2*. We also confirmed that *cax* mutations recombine freely with the *CMD1* locus (data not shown).

Growth characteristics and morphological properties of the *cax* mutants: To further analyze the *cax* mutations, we constructed *cax* strains harboring the wild-type *CMD1* gene integrated in the chromosome in place of *cmd1-226*. We found that *cax2-2* and *cax3-1* grew poorly at 37° (Table 7). The same mutants simultaneously exhibited a calcium-sensitive phenotype. *cax4-1* hardly grew on plates containing 1 μg/ml FK506, a drug that inhibits the activity of calmodulin-dependent phosphoprotein phosphatase, calcineurin (Kunz and Hall 1993). Because *cax2-2*, *cax3-1*, and *cax5-1* cells looked fragile when examined by phase-contrast microscopy, we assayed cell lysis in these cells with a simple plate overlay assay. We found that alkaline phosphatase was easily leaked from cells of *cax2-2*, *cax3-1*, and *cax5-1* at 23° (Table 7). Among the lytic mutants, the temperature-sensitive phenotype of *cax2-2* and *cax3-1* was suppressed by addition of 1 m sorbitol (data not shown). These results suggest that Cax2p, Cax3p, and Cax5p are involved in the mainte-

TABLE 6

Properties of Phe92→X *cmd1* mutations

Allele	Growth phenotype[a]	Viability of *cmd1 myo2-66* double mutants[b]	Small-budded cells with polarized actin network[c] (%)
Wild type	Ts^+		91.9
cmd1-226 F92A	Ts^-	−	9.6
cmd1-376 F92V	Ts^+	+	91.3
cmd1-377 F92I	Ts^+	+	90.8
cmd1-378 F92L	Ts^+	+	93.8
cmd1-379 F92W	Ts^+	+	87.0
cmd1-380 F92M	Ts^+	−	88.2
cmd1-381 F92C	Ts^+	+	89.8
cmd1-382 F92Y	Ts^+	+	94.0

[a] Growth of the cells was assessed at 23° or 38°. Ts^+, wild-type growth; Ts^-, high temperature-sensitive growth.
[b] The *cmd1 myo2-66* double mutants carrying the wild-type calmodulin on a *URA3* plasmid were constructed as described in materials and methods. Their growth on FOA is expressed as follows: −, inviable on FOA; +, viable on FOA.
[c] Actin morphologies of log-phase cells at 33° were observed by staining with phalloidin. More than 180 small-budded cells were scored for the presence of polarized actin patches.

nance of osmotic integrity. Several cells of *cax2*, *cax3*, and *cax5* appeared larger than wild-type cells in the presence (Figure 6, D, E, and G) or absence of 1 m sorbitol (data not shown).

We examined the morphology of the actin cytoskeleton in wild-type, *myo2*, and *cax* cells. To avoid the secondary effect caused by cell lysis, we used medium containing osmotic stabilizer (1 m sorbitol) for *cax2*, *cax3*, and *cax5* cells. In a small-budded, wild-type cell, actin patches were mostly concentrated at the bud (only one or two actin patches were seen in most of the mother cells, Figure 7A), and actin cables were aligned along the axis of the formation of the bud (Figure 6A). Decreased polarity of actin patches and faint actin cables were observed in *myo2* and all *cax* mutants. The percentage of *cax* and *myo2* cells with three or more actin patches in mother cells is 3–4.5-fold larger than in wild-type cells (Figure 7A). The percentage of budded cells that lost actin spot polarization was much larger in *myo2* mutants, and slightly larger in *cax1*, *cax2*, *cax3*, *cax4*, and *cax5* mutants than in a wild-type strain (Figure 7B).

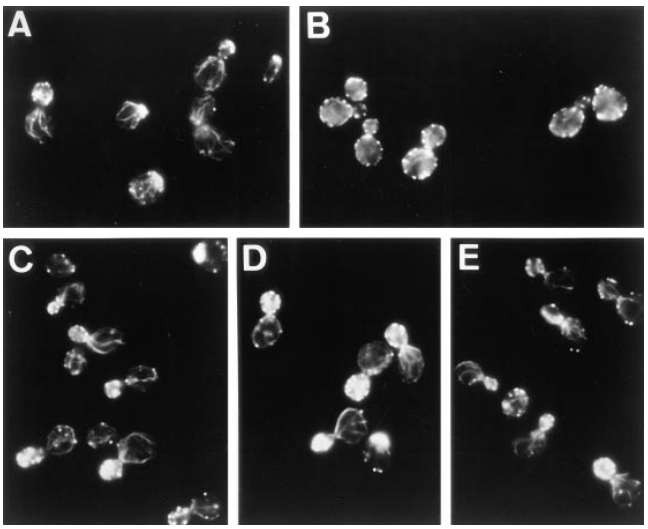

Figure 4.—Actin morphologies of the Phe92→X calmodulin replacement mutants. Cells of the replacement mutants were shifted from 27° to 33° for 170 min and then stained with rhodamine phalloidin. Strains: (A) YOC200 (wild type), (B) YOC226 (*cmd1-226*: F92A), (C) YOC376 (*cmd1-376*: F92V), (D) YOC379 (*cmd1-379*: F92W), and (E) YOC380 (*cmd1-380*: F92M).

Synthetic lethal interaction between *cax* and *cmd1* mutations: The *cax* mutations showed synthetic lethal interaction with *cmd1-226*. To test for allele specificity, we examined synthetic lethality with other calmodulin mutations. For this experiment, we used *cmd1-228* (defective in calmodulin localization at the bud tip), *cmd1-233* (defective in bud emergence), and *cmd1-239* (defective in nuclear division). We revealed (Table 8) that *cax1-1* exhibited synthetic lethal interaction only with *cmd1A* (*cmd1-226*). *cax4-1* showed synthetic lethal interaction with *cmd1A* (*cmd1-226*) and *cmd1B* (*cmd1-228*). *cax5-1* showed synthetic lethal interaction with *cmd1A* and *cmd1D* (*cmd1-233*). *cax2-2* and *cax3-1* showed synthetic lethal interaction with *cmd1A*, *cmd1B*, and *cmd1D*. None of the *cax* mutations showed synthetic lethal interaction with *cmd1C* (*cmd1-239*), which affects nuclear division. These results suggest that Cax1p plays some crucial roles in the function of Myo2p. Because *cmd1A*, *cmd1B*, and *cmd1D* seem to be defective in cell polarity, Cax2p, Cax3p, Cax4p, and Cax5p may have overlapped or diverse functions in the establishment or maintenance of cell polarity.

***CAX1* was identical to *ANP1*/*GEM3*/*MCD2*:** The *CAX1* was cloned, which complemented the FOA sensitivity of the *cax1* mutant. Deletion analysis and subcloning

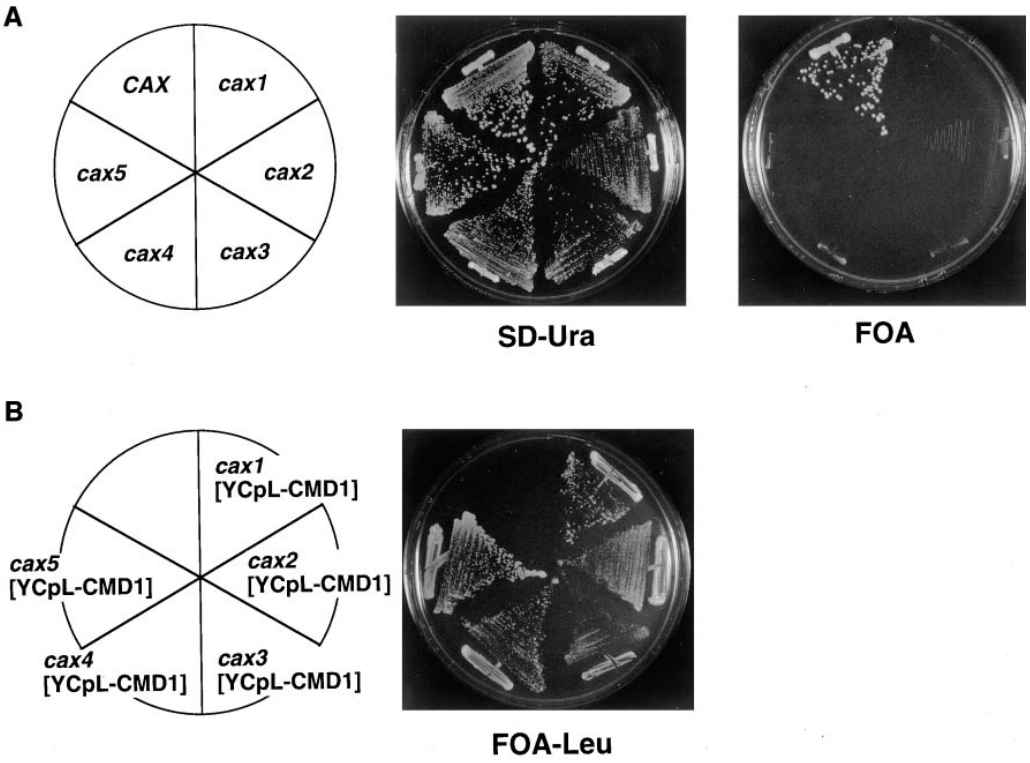

Figure 5.—Growth of *cax* mutants on FOA plates. (A) *cax* mutants fail to lose the wild-type *CMD1* gene on a *URA3* plasmid. *cax* mutant cells were streaked on an FOA plate and incubated at 23° for 5 days. Growth on a permissive medium (SD-Ura) was tested as a control. The strains used are as follows: YOC1119 (*CAX*), YOC1120 (*cax1-1*), YOC1126 (*cax2-1*), YOC1130 (*cax3-1*), YOC1132 (*cax4-1*), and YOC1134 (*cax5-1*). (B) Growth of *cax* mutants does not depend on *URA3*. *cax* cells were transformed with YCpL-CMD1, streaked on an FOA-Leu plate, and incubated for 5 days. The *cax* strains used are the same as described in A.

were used to identify the minimum complementing region. Linkage of the genomic copy of the cloned DNA to *CAX1* was demonstrated (see materials and methods).

The *CAX1* gene was found to be identical to *ANP1/GEM3/MCD2* (amino nitrophenol propandiol resistance gene/Golgi enzyme maintenance gene/multinucleated cells with morphogenetic defects). Deletion of the gene confers sensitivity to amino nitrophenol propandiol (McKnight *et al.* 1981), improper Golgi function, defects in secretion, altered protein glycosylation (Chapman and Munro 1994; Sipos *et al.* 1995), and resistance to sodium orthovanadate (Kanik-Ennulat *et al.* 1995). Mondésert *et al.* (1997) isolated *ANP1* in a screen for mutants defective specifically in polarized growth, but with no drastic impairment in actin cytoskeleton. In contrast, we did detect obvious impairment in actin spot polarization in *cax1* (Figures 6C and 7), although this impairment is not as strong as that observed in mutants of actin-binding proteins. The other phenotypes of *anp1* deletion cells include killer toxin resistance and flocculation, suggestive of defective cell walls (Chapman and Munro 1994). Our *cax1-1* mutant exhibited consistent resistance to sodium orthovanadate and flocculation (data not shown).

To examine whether deletion of *ANP1/CAX1* results in synthetic lethal interaction with *cmd1-226*, we created a strain harboring a deletion mutation of *CAX1*, *cmd1-226*, and the wild-type *CMD1* gene on a *URA3*-marked plasmid. We found that the strain was unable to grow on FOA medium, indicating that the *CAX1* deletion

TABLE 7

Growth phenotypes of the *cax* cells

Phenotype	*cax1*	*cax2*	*cax3*	*cax4*	*cax5*	Wild type
Growth at 23°	+	+	+	+	+	+
Growth at 37°	+	−	−	+	+	+
Growth on 200 mm $CaCl_2$	+	−	−	+	+	+
Growth on 1 μg/ml FK506	+	+	+	−	+	+
Cell lysis	No lysis	Lysis	Lysis	No lysis	Lysis	No lysis

Cells of wild type (YOC1170), *cax1-1* (YOC1139), *cax2-2* (YOC1140), *cax3-1* (YOC1141), *cax4-1* (YOC1142), and *cax5-1* (YOC1143) were scored for their growth on YPD (23° and 37°), YPD + 200 mm $CaCl_2$ (23°), and YPD + 1 μg/ml FK506 (23°). +, wild-type growth; −, poor growth. For the cell lysis assay, cells were incubated on YPD at 23° for 3 days and then stained with alkaline phosphatase substrate. Lysis, cells turned blue; No lysis, cells remained white.

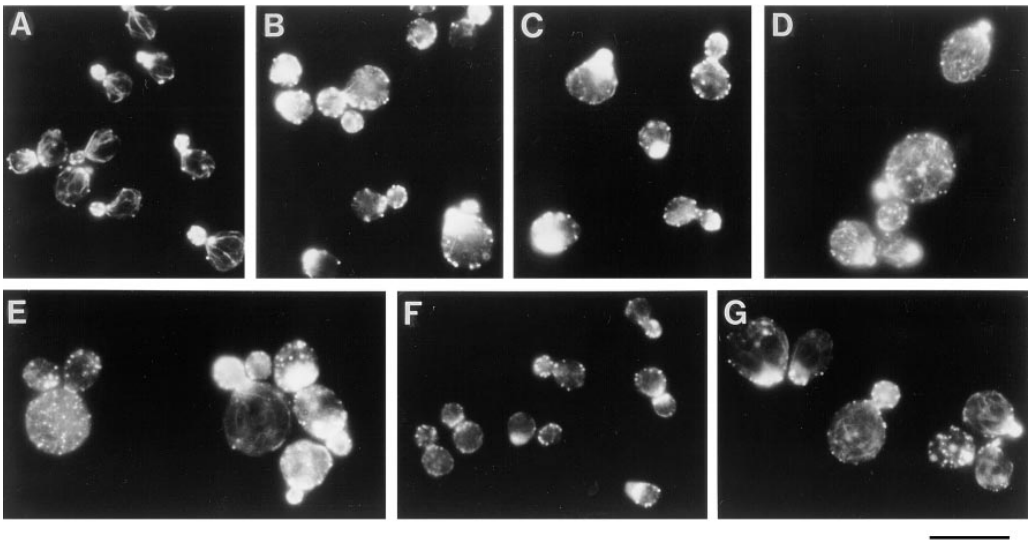

Figure 6.—All *cax* strains have defects in actin organization at 25°. Log-phase cells were fixed and stained with rhodamine phalloidin. Wild-type, *myo2-66*, *cax1-1*, and *cax4-1* cells were cultured in YPD. *cax2-2*, *cax3-1*, and *cax5-1* cells were cultured in YPD + 1 m sorbitol. The strains used are as follows: (A) YOC1170 (wild type), (B) YOC1102 (*myo2-66*), (C) YOC1139 (*cax1-1*), (D) YOC1140 (*cax2-2*), (E) YOC1141 (*cax3-1*), (F) YOC1142 (*cax4-1*), and (G) YOC1143 (*cax5-1*). Bar, 10 μm.

combined with *cmd1-226* results in inviability. Next, synthetic lethal interaction of the deletion was examined with *myo2-66*. We created a strain harboring a *CAX1* deletion, *myo2-66*, and the wild-type *MYO2* gene on a *URA3*-marked plasmid and found that the strain was unable to grow on FOA medium. Furthermore, we confirmed by tetrad analysis that most of the Δ*cax1 myo2-66* cells were inviable. We conclude that deletion of *ANP1*/*CAX1* shows a synthetic growth defect with *myo2-66*.

We found that *CAX4* encodes a novel protein with no significant homology to any other known proteins (the ORF name is YGR036C). Deletion of *CAX4* showed FK506 sensitivity and defects in actin organization, which are phenotypes similar to the *cax4-1* strain (M. Sekiya and Y. Ohya, unpublished results). The *CAX5* gene was found to be identical to *MNN10*/*SLC2*/*BED1* (mannan defective/synthetic lethality with *cap2*/bud emergence delay). The gene shows significant homology to galactosyl transferase. The *slc2* mutant has defects in actin cytoskeleton (Karpova *et al.* 1993). *cax5-1*, as well as *mnn10* (Ballou *et al.* 1991), showed resistance to orthovanadate (data not shown).

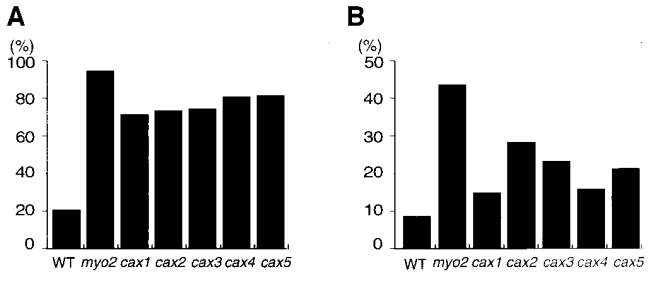

Figure 7.—Morphological properties of *cax* cells. More than 150 small-budded cells were scored for each mutant at 25°. The strains and culture conditions used were the same as described in Figure 6. (A) Percentage of small-budded cells that have three or more actin patches in mother cells. (B) Percentage of budded cells that have lost actin spot polarization.

DISCUSSION

Synthetic lethality with the complementing calmodulin mutations: Synthetic lethal interaction is often observed between mutations in genes that functionally interact. Based on the assumption that each intragenic complementation group of calmodulin mutations results in a single functional defect, allele-specific genetic interaction with the complementing calmodulin mutations seemed likely to serve as a good tool for the dissection of the individual cellular processes regulated by calmodulin.

We found here that among the complementing mutations, only *cmd1-226* (F92A) exhibits synthetic lethal interaction with a *myo2* mutation. A plausible explanation for this result is that among the mutant calmodulins, only Cmd1-226p loses binding activity to Myo2p. Biochemical analysis supported this model, as we found that Cmd1-226p is severely defective in binding to Myo2p, compared to the other calmodulins. The overall structure of Cmd1-226p seems not to be altered by biotinylation because Cmd1-226p was able to bind to another CaM-binding protein, Cna2p (Ca^{2+}/calmodulin-dependent protein phosphatase), as strongly as wild-type calmodulin (H. Okano and Y. Ohya, unpublished results).

The *cmd1-233* (*cmd1D*) mutation does not enhance the temperature-sensitive phenotype of *myo2-66*, but *cmd1-233* and *myo2-66* show a synthetic growth defect at 23° (Table 4). Examination by gel overlay assay indicates that Cmd1-233p physically interacts with Myo2p (Figure 2B). Our explanation is that an unidentified calmodulin target(s) uncoupled by *cmd1-233* exacerbates the *myo2* phenotype. Another possibility is that Cmd1-233p, as

TABLE 8

Synthetic lethal interaciton between *cax* and *cmd1* mutations

Strains crossed	Viability of the double mutants on FOA plates: Alive	Dead	Conclusion
cax1 × *cmd1-228*			
YOC1120 × YOC1178	12	1[a]	Not SL
cax1 × *cmd1-233*			
YOC1120 × YOC1180	14	0	Not SL
cax1 × *cmd1-239*			
YOC1120 × YOC1181	22	0	Not SL
cax2 × *cmd1-228*			
YOC1129 × YOC1172	0	12	SL
cax2 × *cmd1-233*			
YOC1129 × YOC1174	0	5	SL
cax2 × *cmd1-239*			
YOC1129 × YOC1175	18	0	Not SL
cax3 × *cmd1-228*			
YOC1130 × YOC1178	0	26	SL
cax3 × *cmd1-233*			
YOC1130 × YOC1180	0	24	SL
cax3 × *cmd1-239*			
YOC1130 × YOC1181	13	0	Not SL
cax4 × *cmd1-228*			
YOC1132 × YOC1178	0	13	SL
cax4 × *cmd1-233*			
YOC1132 × YOC1180	17	1[a]	Not SL
cax4 × *cmd1-239*			
YOC1132 × YOC1181	15	0	Not SL
cax5 × *cmd1-228*			
YOC1135 × YOC1172	20	0	Not SL
cax5 × *cmd1-233*			
YOC1135 × YOC1174	0	18	SL
cax5 × *cmd1-239*			
YOC1135 × YOC1175	19	0	Not SL

SL, *cax* and *cmd1* (*cmd1-228*, *cmd1-233*, or *cmd1-239*) are predicted to be synthetic lethal; Not SL, *cax* and *cmd1* are not predicted to be synthetic lethal.

[a] The double-mutant strain observed most likely resulted from the acquisition of a spontaneous mutation.

The crosses were made between *cax* strains (*cmd1-226::HIS3 cax* [pYSLU1-CMD1] and *cmd1-228*, *cmd1-233*, or *cmd1-239* strains (*cmd1::LEU2*). As for *cax1* and *cax5*, segregants with the vandate-resistant (see results) Leu^+ and Ura^+ phenotypes were assigned for the double mutants. As for *cax2* and *cax3*, segregants with the calcium-sensitive Leu^+ and Ura^+ phenotypes were assigned for the double mutants. As for *cax4*, segregants with the FK506-sensitive Leu^+ and Ura^+ phenotypes were assigned for the double mutants. The segregants were incubated on FOA plates for 7 days. Their viability on the plate was determined.

yet less severely than Cmd1-226p, causes defects of the Myo2p function. Because we found no synthetic growth defect between *myo2-66* and *cmd1-231* that belongs to the same complementation group of *cmd1-233(cmd1D)*, we think that another essential target uncoupled by *cmd1D* is involved in bud emergence.

Amino acid requirement at position Phe92 of calmodulin: In this study, we demonstrate that Phe92 of yeast calmodulin is essential for the Myo2p–Cmd1p interaction. As reported previously, the target recognition by calmodulin is mainly stabilized by hydrophobic interaction (Ikura *et al.* 1992). Phe92, which is perfectly conserved among eukaryote calmodulins, is one of the residues involved in the interaction. Although the amino acid sequence of yeast calmodulin is only 60% identical to the sequences of other eukaryote calmodulins, functional importance of Phe92 in target recognition seems to be well conserved during evolution. Only hydrophobic and aromatic residues are consistently allowed at Phe92 (Table 5).

Among the several replacement mutants at position 92, only the phenylalanine-to-alanine change results in a temperature-sensitive phenotype. This was an unexpected result, because all tryptophan replacements con-

structed by random replacement mutagenesis showed a temperature-sensitive phenotype. We assume that additional mutations at positions 89–91 affect the temperature-sensitive phenotypes. This result illustrates, in a striking way, that random replacement methods and single-residue replacements do not give the same information, just as was found previously in the case of bacterial β-lactamase (Palzkill and Botstein 1992a,b).

Synthetic lethal interaction between *cmd1-380* (F92M) and *myo2-66* suggests that the F92M substitution of calmodulin alters the Myo2p-Cmd1p interaction. Because the actin morphology of *cmd1-380* cells was indistinguishable from that of wild-type cells (Figure 4E), it is likely that the *cmd1-380* mutation causes a slight defect of the Cmd1p-Myo2p interaction. In the gel overlay assay experiment, however, we observed no obvious impairment in the binding of Cmd1-380p to the Myo2p fusion. Because the gel overlay assay was performed under the condition where the concentration of calmodulin molecule is much higher than *in vivo*, one possibility is that F92M calmodulin *in vivo* might have a defect in binding to Myo2p. Another possibility is that the binding of F92M calmodulin to different IQ motifs might be decreased. Methionine contains a relatively long hydrophobic side chain compared to other nonaromatic hydrophobic residues. Introduction of methionine at position 92 possibly alters the conformation of the hydrophobic surface of calmodulin that is involved in target recognition.

Function of Myo2p: Myo2p has recently been identified as a member of the class V myosins (Johnston *et al.* 1991). Other members include p190 from vertebrate brain (Espindola *et al.* 1992), *dilute* from mouse (Mercer *et al.* 1991), and Myo4p from yeast (Haarer *et al.* 1994). Several observations implicated myosin V in polarized vesicle transport. *dilute* is involved in polarized transport of pigment-containing melanosomes (Mercer *et al.* 1991). *dilute* is also involved in the transport of smooth endoplasmic reticulum membranes in neuronal dendrites (Takagishi *et al.* 1996).

In yeast, the *myo2-66* mutant arrests as an unbudded cell with a large number of accumulated intracellular vesicles (Johnston *et al.* 1991; Govindan *et al.* 1995). Because actin is thought to be required for polarized secretion in yeast (Novick and Botstein 1985), it is possible that Myo2p serves as an actin-based vesicle motor. However, nobody has yet observed accumulation of secretory product within *myo2-66* cells.

Here we present both genetic and biochemical evidence indicating that the *cmd1-226* mutation results in loss of binding with Myo2p. This is consistent with the observation that *myo2-66* shows defects in actin organization, even at the permissive temperature (Johnston *et al.* 1991). Because Myo2p is localized correctly in a *cmd1-226* mutant (Figure 3C), binding of calmodulin to Myo2p is not necessary for intracellular localization of Myo2p, but is probably necessary for the Myo2p activity in organizing the actin cytoskeleton.

Several possibilities can be envisioned for the Myo2p function in actin organization. Myo2p itself may be involved in localizing or moving the actin cytoskeleton toward the growing tip. Alternatively, cargoes in secretory vesicles driven by Myo2p may anchor or stabilize the actin cytoskeleton. Myo2p might be capable of cross-linking actin because of the predicted and demonstrated ability of class V myosins to dimerize (Cheney *et al.* 1993). There is growing evidence suggesting that myosins regulate the actin network in yeast and other organisms. For example, loss of myosin I function in yeast results in defective actin organization (Geli and Riezman 1996; Goodson *et al.* 1996). It was suggested that 95F myosin (a class VI unconventional myosin) in *Drosophila* may be involved in the formation of actin furrows through the transport of cytoplasmic components (Mermall and Miller 1995).

Isolation and characterization of *cax* mutations: To search for other components that function together with calmodulin and Myo2p, we used a synthetic lethal screen beginning with *cmd1-226*. One property common to *myo2-66* and all the *cax* mutants is altered morphology of actin cytoskeleton, suggesting that all the *CAX* genes somehow function in the regulation of actin organization.

From the similarity of the phenotype between *cax1-1* and *myo2-66* strains, we think that Anp1p/Cax1p is likely to play a crucial role in the regulation of the actin network through calmodulin and Myo2p. Because deletion of *ANP1/CAX1* shows a synthetic growth defect with *myo2-66*, Anp1p/Cax1p may share common functions with Myo2p. Anp1p is known to be involved in protein glycosylation. Given that Myo2p is involved in polarized secretion and that protein glycosylation occurs in the secretory process, one possibility is that the performance of Anp1p function depends on the secretory process regulated by Myo2p. We have found evidence that many late secretory mutations cause, as part of their secretion phenotypes, disorganization of the actin cytoskeleton (Mulholland *et al.* 1997).

Another possibility is that Anp1p might regulate the components of the actin cytoskeleton simply because proper glycosylation of proteins might be required for actin organization. We are attracted to this idea because (1) invertase was not accumulated in the *cmd1-226* (data not shown) and *myo2-66* cells (Govindan *et al.* 1995), and (2) *cmd1-226*, *myo2-66*, and *anp1/cax1* share a common feature of defects in actin organization.

In conclusion, examination of genetic interaction with complementing calmodulin mutations appears to be an effective approach to identify the target that is severely uncoupled by *cmd1A*. By using this approach, we found evidence suggesting that novel components, including Myo2p and Anp1p, are involved in the process of actin organization regulated by calmodulin.

We wish to thank G. C. Johnston for providing us with the *myo2-66* strain. We also thank S. S. Brown for providing us with the anti-Myo2 antibody. This work was supported by a grant for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (Y.O.), Takeda Science Foundation (Y.O.), Kowa Life Science Foundation (Y.O.), National Institutes of Health grant GM-46406 (D.B.), and a grant from the Japan Society for the Promotion of Science for Young Scientists (M.S.-K.).

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Communicating editor: M. D. Rose