# Structure-Based Systematic Isolation of Conditional-Lethal Mutations in the Single Yeast Calmodulin Gene

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ABSTRACT

Conditional-lethal mutations of the single calmodulin gene in *Saccharomyces cerevisiae* have been very difficult to isolate by random and systematic methods, despite the fact that deletions cause recessive lethality. We report here the isolation of numerous conditional-lethal mutants that were recovered by systematically altering phenylalanine residues. The phenylalanine residues of calmodulin were implicated in function both by structural studies of calmodulin bound to target peptides and by their extraordinary conservation in evolution. Seven single and 26 multiple Phe → Ala mutations were constructed. Mutant phenotypes were examined in a haploid *cmd1* disrupted strain under three conditions: single copy, low copy, and overexpressed. Whereas all but one of the single mutations caused no obvious phenotype, most of the multiple mutations caused obvious growth phenotypes. Five were lethal, 6 were lethal only in synthetic medium, 13 were temperature-sensitive lethal and 2 had no discernible phenotypic consequences. Overexpression of some of the mutant genes restored the phenotype to nearly wild type. Several temperature-sensitive calmodulin mutations were suppressed by elevated concentration of $CaCl_2$ in the medium. Mutant calmodulin protein was detected at normal levels in extracts of most of the lethal mutant cells, suggesting that the deleterious phenotypes were due to loss of the calmodulin function and not protein instability. Analysis of diploid strains heterozygous for all combinations of *cmd1-ts* alleles revealed four intragenic complementation groups. The contributions of individual phe→ala changes to mutant phenotypes support the idea of internal functional redundancy in the symmetrical calmodulin protein molecule. These results suggest that the several phenylalanine residues in calmodulin are required to different extents in different combinations in order to carry out each of the several essential tasks.

CALMODULIN is a $Ca^{2+}$-binding protein found in all eukaryotic cells. Calmodulin is implicated in a great variety of $Ca^{2+}$-dependent cellular processes, including signal transduction, motility, secretion, control of the cell cycle, and regulation of glycolysis as well as other aspects of cell metabolism. Calmodulin is known to regulate more than 20 enzymes and to bind several cytoskeletal components (COHEN and KLEE 1988). Presumably the great variety of different functions of calmodulin is expressed through orchestrated interactions with the many calmodulin-binding proteins.

The three dimensional structure of calmodulin shows a dumbbell-shaped molecule consisting of two structurally similar globular lobes (corresponding roughly to the N-terminal and C-terminal halves of the amino acid sequence) connected by an eight turn central α-helix (BABU *et al.* 1988; TAYLOR *et al.* 1991; CHATTOPADHYAYA *et al.* 1992; RAO *et al.* 1993). Upon binding its target peptide and $Ca^{2+}$, the central α-helix bends to form a jaw-like structure–the two lobes clamping down on the target peptide (IKURA *et al.* 1992; MEADOR *et al.* 1992). Each of the lobes contains a very large hydrophobic interaction surface; this may help to explain why calmodulin forms complexes with so many dissimilar (at the level of primary sequence identity) calmodulin-binding proteins (MEADOR *et al.* 1992).

The budding yeast *Saccharomyces cerevisiae* has proven to be an excellent organism for studying the function of calmodulin in cell proliferation (OHYA and ANRAKU 1992; DAVIS 1992a). There is only one structural gene (*CMD1*) encoding this protein which is essential for cell growth (DAVIS *et al.* 1986). The amino acid sequence of yeast calmodulin is 60% identical to that of mammalian calmodulin. Although the sequence of calmodulin is somewhat less conserved than those of the cytoskeletal proteins actin and tubulin (BOTSTEIN 1991), functional replacement of yeast calmodulin by expression of vertebrate calmodulin (OHYA and ANRAKU 1989a; DAVIS and THORNER 1989) indicates conservation of the essential activities required for cell growth.

Despite considerable effort, it has not been possible to find simple point mutations in the *CMD1* gene with conditional-lethal phenotypes. Instead, three more complicated genetic circumstances that result in conditional lethality have been used to try to characterize the function of calmodulin. One involves a strain in which the only functional calmodulin gene is driven by a *GAL1* promoter (OHYA and ANRAKU 1989b); another involves a

double-mutant (*cmd1-1*) that consists of two sequence changes (I100N and E104V), both required to produce a temperature-sensitive growth phenotype (Davis 1992b); the third involves an overproduced C-terminal fragment of the *CMD1* gene (*cmd1-101*) (Sun *et al.* 1991). When expression of the intact *CMD1* driven by the *GAL1* promoter is turned off, the yeast cell cycle eventually arrests at nuclear division; most cells are budded and the DNA has apparently been duplicated (Ohya and Anraku 1989a,b). The *cmd1-1* double mutant loses viability during nuclear division, although the terminal phenotype is not homogeneous (Davis 1992b). Cells with the *cmd1-101* mutation, consisting of a C-terminal fragment ("half-calmodulin") expressed at high level by the *GAL1* promoter (Sun *et al.* 1992), show a $Ca^{2+}$-suppressible temperature-sensitive phenotype (Sun *et al.* 1991). These cells arrest with apparently duplicated DNA but with a single spindle pole body (Sun *et al.* 1992).

The relatively small number of useful conditional circumstances, their complexity, and the ensuing confusion about the function(s) of calmodulin, stimulated us to undertake systematic mutagenesis of this gene. Conventional methods of isolating temperature-sensitive *cmd1* mutations at random yielded an unexpectedly small number of conditional-lethal mutations. Davis (1992b) used a "plasmid shuffle" technique to screen random mutations in the *CMD1* gene, and isolated only one temperature sensitive strain containing the two mutations that comprise *cmd1-1* from 35,000 mutagenized colonies. Other schemes of random mutagenesis, although quite useful with other proteins (Holm *et al.* 1985; Huffaker *et al.* 1988; Schatz *et al.* 1988), had repeatedly failed to yield conditional-lethal *cmd1* mutants (G. H. Sun, Y. Ohya and Y. Anraku, unpublished result).

Classical mutagenesis studies with the single yeast actin gene (*ACT1*) had also yielded relatively few useful conditional-lethal alleles. Unlike actin, however, classical mutagenic strategies with calmodulin failed to produce even simple unconditional lethal alleles; although deletion was clearly lethal, point mutations apparently never sufficed to eliminate the essential function(s) of calmodulin. Recently systematic site-directed mutagenesis, in which clusters of charged residues were changed to alanine residues yielded a surprisingly large number of new lethal and conditional-lethal alleles in *ACT1* (Wertman *et al.* 1992). A similar approach yielded many new alleles in the single β-tubulin (*TUB2*) gene (Reijo *et al.* 1994). However, application of the same technology to the *CMD1* gene again failed, most surprisingly, to yield conditional-lethal or lethal alleles (Y. Ohya and D. Botstein, unpublished result).

For two reasons we then focused our site-directed mutagenesis effort on phenylalanine residues in the yeast calmodulin sequence. First, phenylalanine residues are particularly well conserved in calmodulins (Cohen and Klee 1988): the phenylalanine residues in the yeast sequence appear in the same positions as in the vertebrate sequences. None of the other 19 amino acid residues shows this degree of exact conservation. Given that vertebrate calmodulin can substitute for essential function(s) of yeast calmodulin (Ohya and Anraku 1989a; Davis and Thorner 1989), conserved phenylalanine residues in calmodulin seemed likely to play fundamental roles for cell growth. Second, the three-dimensional structure of calmodulin suggests that the phenylalanine residues are likely to be important for interaction with other proteins. Phenylalanine residues form clusters that project from the hydrophobic interaction surface in both in the N-terminal lobe and the C-terminal lobe (Babu *et al.* 1988; Ikura *et al.* 1991). Further, the NMR structure of calmodulin in solution and that of a calmodulin-binding peptide complex (Ikura *et al.* 1992) shows that seven of the eight phenylalanine residues interact with the target peptide.

We present here the results of systematic phenylalanine to alanine mutagenesis of *CMD1*, the yeast calmodulin gene. After introduction of mutations by site-directed mutagenesis, we assessed the growth phenotype across a broad temperature range. We recovered 7 single mutations and 26 multiple mutations. One of the seven single mutations, and 24 of the 26 multiple mutations caused a lethal or conditional-lethal phenotype.

## MATERIALS AND METHODS

**Yeast media and genetic methods:** Yeast growth, complete and minimal media, and tetrad analysis were performed as described by Rose *et al.* (1990). For induction of the *GAL1* promoter, minimal medium containing 2% galactose plus 0.1% sucrose (SGS) and rich media containing 2% galactose plus 0.1% sucrose (YPGS) were used. $Ca^{2+}$-rich medium containing final concentration of 100 mM $CaCl_2$ was made by addition of 5 M $CaCl_2$ into YPD liquid or YPD agar media cooled below 70°. Lithium acetate yeast transformation was performed using a modification (Schiestl and Gietz 1989) of the original method (Ito *et al.* 1983).

**Plasmid construction:** All DNA manipulations were performed according to standard techniques (Sambrook *et al.* 1989). DH5αF′ was used to maintain and propagate plasmids. Restriction endonucleases, T4 polymerase, T4 ligase, T4 polynucleotide kinase were obtained from New England Biolabs. Vent DNA polymerase (New England Biolabs) and Pfu DNA polymerase (Stratagene) were used for polymerase chain reaction (PCR) with DNA thermal cycler (Perkin-Elmer). Purification of DNA fragments was routinely carried out by electrophoresis in agarose followed by treatment with Geneclean (BIO 101, Inc.).

pRB1612 (pRSCAM101) was made as follows: a 2.0-kb *CMD1*-containing genomic *Sal*I-*Bam*HI fragment from pCAM106 (Y. Ohya, unpublished) was inserted into the *Sal*I-*Bam*HI gap of the *CEN, URA3* plasmid pRS316 (Sikorsky and Hieter 1989).

pRB1613 (YIpCAMT106) was used for disruption of *CMD1* with *TRP1* (*cmd1-Δ1::TRP1*; Sun *et al.* 1991).

pRB1614 (pJJ281-HIS3) was made by insertion of *Bam*HI *HIS3*-containing fragment from pJJ217 (Jones and Prakash 1990) into *Eco*RV sites of pJJ281 (Jones and Prakash 1990) after fill-in reaction, and used to change the markers from *TRP1 his3* to *trp1 HIS3*.

**TABLE 1**

**Primers used for PCR-based mutagenesis**

| 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(F12)$^+$ | TG TCT TCG AAT CTT ACC GAA GAA CAA ATT GCT GAA GCC AAA GAA GCC TTT GCC |
| CMD1(F16F19)$^+$ | TG TCT TCG AAT CTT ACC GAA GAA CAA ATT GCT GAA TTC AAA GAA GCC GCT GCC CTC GCT GAT AAA GAT AAC AAT |
| CMD1(F12F16F19)$^+$ | TG TCT TCG AAT CTT ACC GAA GAA CAA ATT GCT GAA GCC AAA GAA GCC GCT GCC CTC GCT GAT AAA GAT AAC AAT |
| CMD1(F65)$^+$ | AC CAT CAA ATC GAA GCT AGT GAA TTT TTG GCT CTG ATG T |
| CMD1(F65)$^-$ | A CAT CAG AGC CAA AAA TTC ACT AGC TTC GAT TTG ATG GT |
| CMD1(F68)$^+$ | AC CAT CAA ATC GAA TTT AGT GAA GCT TTG GCT CTG ATG T |
| CMD1(F68)$^-$ | A CAT CAG AGC CAA AGC TTC ACT AAA TTC GAT TTG ATG GT |
| CMD1(F65F68)$^+$ | AC CAT CAA ATC GAA GCT AGT GAA GCT TTG GCT CTG ATG T |
| CMD1(F65F68)$^-$ | A CAT CAG AGC CAA AGC TTC ACT AGC TTC GAT TTG ATG GT |
| CMD1(F89)$^+$ | AA CTA CTA GAA GCT GCT AAA GTA TTC GAT AAG AAC GGT G |
| CMD1(F89)$^-$ | C ACC GTT CTT ATC GAA TAC TTT AGC AGC TTC TAG TAG TT |
| CMD1(F92)$^+$ | AA CTA CTA GAA GCT TTT AAA GTA GCC GAT AAG AAC GGT G |
| CMD1(F92)$^-$ | C ACC GTT CTT ATC GGC TAC TTT AAA AGC TTC TAG TAG TT |
| CMD1(F89F92)$^+$ | AA CTA CTA GAA GCT GCT AAA GTA GCC GAT AAG AAC GGT G |
| CMD1(F89F92)$^-$ | C ACC GTT CTT ATC GGC TAC TTT AGC AGC TTC TAG TAG TT |
| CMD1(F140)$^+$ | TC AAC ATT CAA CAA GCC GCT GCT TTG TTA T |
| CMD1(F140)$^-$ | A TAA CAA AGC AGC GGC TTG TTG AAT GTT GA |

"+" and "−" mean the sense strand and anti-sense strand, respectively. Nucleotide changes are underlined.

prb1615 (YCpTG5189T) is a *CEN* plasmid containing a *GAL1* promoter, a *CMK1* terminator and a *TRP1* marker and was made as follows: an 0.8-kb *Bam*HI-*Eco*RI fragment of the *GAL1* promoter (Ohya and Anraku 1989a,b), a 34-bp *Eco*RI-*Hin*dIII oligonucleotide synthesized cassette (mixture of 5′AATTCAATGTCTTCGAATCTTACGCATGCA3′ and 5′AGCTTGCATGCGTAAGATTCGAAGACATTG3′; it contains *Eco*RI, *Bst*BI, *Sph*I and *Hin*dIII sites in this order) and a 0.9-kb *Hin*dIII-*Cla*I fragment of the *CMK1* terminator (Ohya *et al.* 1991) were inserted into *Bam*HI-*Cla*I gap of pRS316 with successive subcloning steps. Then, the whole 1.7-kb *Bam*HI-*Cla*I *GAL1* promoter-*CMK1* terminator fragment was subcloned into the *Bam*HI-*Cla*I gap of pRS315 (Sikorsky and Hieter 1989) to make pRB1615. pRB1615 was designed to overexpress yeast calmodulin after subcloning a PCR-amplified *Bst*BI-*Sph*I fragment of *CMD1*.

pRB1616 (YCpTcmd1p) is a *CEN* plasmid containing a *CMD1* promoter, the *CMK1* terminator and the *TRP1* marker, constructed as follows: the *CMD1* promoter fragment (212 bp) was PCR-amplified with 5′CGCTTCGAAGACATTGTACTTTTTTATTTG3′ and 5′CCCGGATCCTGTATTTATATTTTCGTGTAC3′ as primers and pRB1612 as a template. These primers were designed so that (1) a *Bam*HI site was placed upstream of the promoter and (2) a *Bst*BI site was introduced just after the translational start without changing the amino acid sequence of calmodulin (Ohya and Anraku 1989b). The presence of a competent promoter on this fragment was confirmed both by its ability to express calmodulin enough to complement the *cmd1* disruption mutation and by direct measurement of the level expression (see Table 4 below). pRB1616 was constructed by insertion of the *Bam*HI-*BstBI* fragment of the PCR-amplified *CMD1* promoter into the *Bam*HI-*BstBI* gap of pRB1615 (a *CEN* plasmid). pRB1616 was designed to test the calmodulin mutant phenotype in a "low copy" condition after subcloning the *Bst*BI-*Sph*I PCR amplified *CMD1* fragment.

pRB1617 (YIpHade3) is an integration plasmid containing a *HIS3* marker inserted into the coding region of *ADE3*. To make it, a 2.4-kb *Sac*II-*Kpn*I fragment of the genomic *ADE3* gene was subcloned into the *Sac*II-*Kpn*I gap of pBluescript KS$^+$ (Stratagene). Then, a 1.5-kb *Sal*I-*Xho*I *HIS3*-containing fragment of pJJ217 (Jones and Prakash 1990) was inserted into the unique *Xho*I site such that *HIS3* is transcribed in the direction opposite to *ADE3*. pRB1617 has advantages as a vehicle for chromosomal integration of the *cmd1* mutations. First, pRB1617 had unique and adjacent *Bam*HI and *Sph*I sites so that the *Bam*HI-*Sph*I *CMD1*-containing fragment was easily inserted to make the *CMD1* integration plasmids. *Sac*II and *Alw*NI (or *Avr*II) could be used for linearization of the plasmid. Second, after transformation of the yeast *ade2* strains, the correct chromosomal integration could be screened by colony color.

pRB1619 (YCpLG-cCaM) is a *CEN* plasmid containing the chicken calmodulin coding sequence under the *GAL1* promoter and a *LEU2* marker. To construct it, a 1.7-kb *Bam*HI-*Sal*I fragment of chicken calmodulin under the *GAL1* promoter was cut out from pGCAM211 (Ohya and Anraku 1989a) and inserted into the *Bam*HI-*Sal*I gap of pRS315 (Sikorsky and Hieter 1989). pRB1619 was used for the measurement of the steady state level of calmodulin in the lethal calmodulin mutants.

**PCR-based mutagenesis:** Introduction of Phe → Ala mutations was performed with PCR-based mutagenesis (Ho *et al.* 1989). We synthesized 19 oligonucleotides (Table 1). CMD1N$^+$, corresponding to the translation start sequence of calmodulin, introduced an artificial *Bst*BI site without changing any amino acid sequence and CMD1C$^-$, corresponding to the 3′-noncoding sequence, contained an *Sph*I site at the end. PCR reactions (Saiki *et al.* 1988) with these primers yielded 656-bp fragments containing *Bst*BI and *Sph*I sites at the end(s) making it easy to subclone into pRB1616.

Long N-terminal primers with N-terminal Phe → Ala utations, CMD1(F12)$^+$, CMD1(F16F19)$^+$ and CMD1(F12F16F19)$^+$, were used for amplification of the *cmd1-221*, *cmd1-222* and *cmd1-228* mutations, respectively, using pRB1612 as a template and CMD1C$^-$ as the other primer. The PCR-amplified fragment was purified by electrophoresis on 0.7% agarose gel, digested with *Sph*I for more than 12 hr, and then digested with *Bst*BI for 2 hr at 55°. The *Bst*BI-*Sph*I fragment was purified again and then subcloned into the *Bst*BI-*Sph*I gap of PRB1616.

Internal mutations were constructed using a two-step PCR method (Ho *et al.* 1989). In the first PCR reaction, both

**TABLE 2**

**Phe→Ala calmodulin mutants constructed in this study**

| CMD1 allele (mutation site) | Strains | | | |
|---|---|---|---|---|
| | Integration | | | |
| | Mata | Matα | Low dose | High dose |
| *cmd1-221* (F12A) | YOC221 | YOC321 | YOC421 | |
| *cmd1-222* (F16A F19A) | YOC222 | YOC322 | YOC422 | |
| *cmd1-223* (F65A) | YOC223 | YOC323 | YOC423 | |
| *cmd1-224* (F68A) | YOC224 | YOC324 | YOC424 | |
| *cmd1-225* (F89A) | YOC225 | YOC325 | YOC425 | |
| *cmd1-226* (F92A) | YOC226 | YOC326 | YOC426 | YOC526 |
| *cmd1-227* (F140A) | YOC227 | YOC327 | YOC427 | |
| *cmd1-228* (F12A F16A F19A) | YOC228 | YOC328 | YOC428 | YOC528 |
| *cmd1-229* (F12A F65A) | YOC229 | YOC329 | YOC429 | |
| *cmd1-230* (F12A F68A) | YOC230 | YOC330 | YOC430 | |
| *cmd1-231* (F12A F89A) | YOC231 | YOC331 | YOC431 | YOC531 |
| *cmd1-232* (F12A F92A) | YOC232 | YOC332 | YOC432 | YOC532 |
| *cmd1-233* (F12A F140A) | YOC233 | YOC333 | YOC433 | YOC533 |
| *cmd1-234* (F16A F19A F65A) | YOC234 | YOC334 | YOC434 | YOC534 |
| *cmd1-235* (F16A F19A F68A) | YOC235 | YOC335 | YOC435 | YOC535 |
| *cmd1-236* (F16A F19A F89A) | YOC236* | YOC336* | YOC436* | YOC536* |
| *cmd1-237* (F16A F19A F92A) | YOC237* | YOC337* | YOC437* | YOC537* |
| *cmd1-238* (F16A F19A F140A) | YOC238* | YOC338* | YOC438* | YOC538* |
| *cmd1-239* (F65A F68A) | YOC239 | YOC339 | YOC439* | YOC539* |
| *cmd1-240* (F65A F89A) | YOC240 | YOC340 | YOC440 | YOC540 |
| *cmd1-241* (F65A F92A) | YOC241* | YOC341* | YOC441* | YOC541* |
| *cmd1-242* (F65A F140A) | YOC242 | YOC342 | YOC442 | YOC542 |
| *cmd1-243* (F68A F89A) | YOC243* | YOC343* | YOC443* | YOC543* |
| *cmd1-244* (F68A F92A) | YOC244* | YOC344* | YOC444* | YOC544* |
| *cmd1-245* (F68A F140A) | YOC245* | YOC345* | YOC445* | YOC545* |
| *cmd1-246* (F89A F92A) | YOC246* | YOC346* | YOC446* | YOC546* |
| *cmd1-247* (F89A F140A) | YOC247 | YOC347 | YOC447 | YOC547 |
| *cmd1-248* (F92A F140A) | YOC248* | YOC348* | YOC448* | YOC548* |
| *cmd1-249* (F16A F19A F65A F68A) | YOC249* | YOC349* | YOC449 | YOC549 |
| *cmd1-250* (F12A F65A F68A) | YOC250 | YOC350 | YOC450 | YOC550 |
| *cmd1-251* (F12A F16A F19A F68A) | YOC251 | YOC351 | YOC451 | YOC551 |
| *cmd1-252* (F12A F16A F19A F65A) | YOC252 | YOC352 | YOC452 | YOC552 |
| *cmd1-253* (F12A F16A F19A F65A F68A) | YOC253* | YOC353* | YOC453 | YOC553 |
| Wild type | YOC200 | YOC300 | YOC400 | YOC500 |

Strains carrying lethal mutations (marked with an asterisk*) also contained pRB1612 which carries wild-type *CMD1* and *URA3*. Integration strains, YOC221-YOC253 and YOC321-YOC353 were constructed from YOC101 (*MAT***a** *ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::TRP1* [pRB1612]) and YOC102 (same as YOC101 except its mating type), respectively. Low dose and high dose strains were derived from YOC103 (*MAT***a** *ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::HIS3* [pRB1612]).

N-terminal fragment and C-terminal fragment were amplified separately and purified after electrophoresis on 15% polyacrylamide (SAMBROOK *et al.* 1989). These two fragments shared at least 18 bp overlapping region containing the Phe → Ala mutations so that in a second PCR reaction, both fragments were mixed and the whole fragment was amplified with $CMD1N^+$ and $CMD1C^-$ as primers. Purification and subcloning of the PCR-amplified fragment was described before. In this way, *cmd1-223*, *cmd1-224*, *cmd1-225*, *cmd1-226*, *cmd1-227* mutations were constructed. Other double and heavily mutagenized mutants were constructed in a similar way using the single mutation plasmids as template.

All mutations were verified by DNA sequencing. Double-stranded DNA sequencing with two sequencing primers (5′TGACCGGAAACTACTGAAC3′, 5′GATGAACGAAATAGATGTTGATGG3′) sufficed to cover the entire coding sequence of calmodulin. 7-Deaza-dGTP sequencing kit (U. S. Biochemical Corp.), and version 2.0 Sequenase (U. S. Biochemical Corp.) were used for sequencing reactions.

A complete set of *cmd1* mutations on a centromere plasmid (pRB1616) were made initially. Subsequently each of the *Bst*BI-*Sph*I fragments containing the mutant *cmd1* coding sequences were subcloned into the *Bst*BI-*Sph*I gap of PRB1615 to make the set of overexpression plasmids. The same fragments were separately subcloned into the *Bam*HI-*Sph*I gap of PRB1617 to make the set of integration plasmids.

**Introduction of the mutations into yeast:** The 2.4-kb *Bam*HI fragment of PRB1613 that contains the *cmd1-Δ1::TRP1* fragment was purified by electrophoresis in agarose and used to transform a yeast diploid strain, YPH501 (SIKORSKY and HIETER 1989). Southern hybridization analysis was conducted to confirm that one copy of *CMD1* was replaced with *cmd1-Δ1::TRP1*. The heterozygous *CMD1/cmd1-Δ1::TRP1* strain was transformed with pRB1612 (*CMD1*, *URA3*), sporulated, and subjected to tetrad analysis. Two segregants harboring the *cmd1-Δ1::TRP1* allele and pRB1612 were selected: YOC101 [*MAT***a** *ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::TRP1* (pRB1612)] and YOC102 [*MATα ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::TRP1* (pRB1612)] were used for parent strains for introduction of the mutations. YOC103 [*MAT***a** *ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::HIS3* (pRB1612)], a derivative of YOC101, was constructed using pRB1614 to replace the *TRP1 his3* markers with *trp1 His3*; this strain was used for introducing the mutations at low copy number.

The series of pRB1617-derived plasmids was used to integrate the mutations into the genome. After digestion of these

plasmids with *Sac*II and *Alw*NI (or *Sac*II and *Avr*II), the *ade3-cmd1-HIS3-ade3* fragments were used to transform YOC101 or YOC102 selecting for the *HIS3* marker. Correct integrants were recognized as white transformants (*ade2 ade3*; see above), and each was confirmed with PCR. Finally, strains that had lost pRB1612 were selected on FOA plates. The resulting Phe → Ala calmodulin mutant strains are listed in Table 2.

**Plasmid loss using fluoro-orotic acid (FOA) plates:** FOA (or FOA-Glc) plates containing 0.2% 5-FOA monohydrate (PCR Inc.) were made according to ROSE *et al.* (1990). FOA-Gal plates were similar except 2% glucose was replaced with 2% galactose and 0.1% sucrose.

Cells bearing the wild-type calmodulin gene on a *URA3* plasmid (pRB1612) and a calmodulin mutation either at the *ade3* locus (integration) or on a *TRP1 CEN*-plasmid (low copy) were fully grown in YPD from single colonies at 25°. The cell suspensions were diluted with FOA medium, incubated at 25° for 8 hr, spotted on the surface of FOA agar plates with a multi-point inoculator and incubated at 25° for 3 days to let Ura3$^-$ cells grow. Cells bearing the wild-type calmodulin gene on a *URA3* plasmid and a calmodulin mutation under the *GAL1* promoter were handled similarly, except that FOA-Gal plates were incubated at 25° for 1 week.

Occasionally we observed spotty FOA resistance where we expected none. In each such case PCR experiments showed that the wild-type *CMD1* gene was retained, indicating that these were the result of events unrelated to calmodulin, such as spontaneous *ura3* mutations or gene conversion.

**Steady-state labeling and immunoprecipitation of calmodulin:** Cells were grown to $2 \times 10^7$ cells/ml in synthetic minimal medium lacking sulfate (ROTHBLATT and SCHEKMAN 1989) and containing 2% galactose, 0.1% sucrose and 100 μM $(NH_4)_2SO_4$. Cells ($4 \times 10^7$) were harvested, washed once with distilled water and resuspended in 500 μl of the same medium containing 2% galactose and 0.1% sucrose. After incubation for 10 min at 30°, 400 μCi [$^{35}$S]$Na_2SO_4$ (ICN) were added. After 30 min, labeling was terminated by addition of equal volumes of ice-cold 20 mM sodium azide. Immunoprecipitation with anti-calmodulin antibody (OHYA *et al.* 1987) was carried out according to ROTHBLATT and SCHEKMAN (1989). Mobility shift of calmodulin by $Ca^{2+}$ was examined by addition of either 100 mM $CaCl_2$ or 100 mM EGTA into all of the solutions used for SDS-PAGE. Samples were heated again at 100° either in the presence of 100 mM $CaCl_2$ or in the presence of 100 mM EGTA, and the equal amount of labeled proteins were subjected to SDS-PAGE (LAEMMLI *et al.* 1970) using a 12.5% polyacrylamide gel. [14C]-labeled Rainbow protein markers (Amersham, England) were used as protein size markers. After electrophoresis, the gels were treated with Amplify (Amersham), dried and exposed to Kodak X-Omat AR film for 1–2 days at −80°. The levels of the wild-type and the mutant calmodulin were determined with PhosphoImager analysis according to the manufacture's manual (Molecular Probes).

**Pulse-chase labeling:** After labeling as above with [$^{35}$S]$Na_2SO_4$ for 10 min, the chase was initiated by the addition of equal volume of 2 × YPGS medium containing 2 mM $(NH_4)_2SO_4$, 0.006% L-cysteine and 0.008% L-methionine. Immediately after initiating the chase, equivalent aliquots were transferred to fresh YPGS or YPGS plus 100 mM $CaCl_2$ media, and chased in the two media at different temperature. Equivalent aliquots were removed at the indicated time point and added to equal volumes of ice-cold 20 mM sodium azide to terminate the pulse and chase. Harvest of the cells, extraction of cell lysate, and immunoprecipitation are described in the previous section. Incorporation of $^{35}$S into the cell lysate was measured with Ready Cap (Beckman), and used for adjusting the cell lysate concentration before electrophoresis.

## RESULTS

**Experimental design:** There are eight phenylalanine residues in the yeast calmodulin sequence: F12, F16, F19, F65, F68, F89, F92 and F140. These Phe residues were replaced systematically with alanine on the assumption that this would eliminate the hydrophobic effect of the side chain beyond the β-carbon without altering the main-chain conformation. All Phe residues were changed individually with the exception of F16 and F19, which are very close to each other in the structure and were changed together. Mutants are referred to below using single-letter code so that the aforementioned double mutation is called F16A, F19A. PCR-based mutagenesis (see MATERIALS AND METHODS) was used to introduce the mutations and every mutation was verified by direct DNA sequencing.

Phenotypes resulting from Phe → Ala calmodulin mutants were assessed under three different expression conditions (Figure 1), namely "integrated," "low dose" and "high dose."

"Integrated" means that the mutant *cmd1* gene was integrated into the genome and thus presumably contains a single copy of the mutant gene driven by its normal promoter. Mutant *cmd1* genes were integrated at the *ADE3* locus of a strain with a *cmd1* deletion (*cmd1-Δ1::TRP1*) and a wild-type *CMD1* gene on a *URA3* plasmid to keep it alive (Figure 1A). We used strains YOC101 or YOC102 (*MAT***a** or *MATα, ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::TRP1* [pRB1612]); each of these forms red colonies (ROMAN 1956). After insertion of the *cmd1-HIS3* fragment into the *ADE3* gene, the cells form white colonies, having lost the ability to make the precursor of the red pigment that accumulates in *ade2* strains (*cf.* KOSHLAND *et al.* 1985). After integration, derivatives that had lost the *URA3-CMD1* plasmid were selected on 5-FOA plates, revealing any recessive phenotype(s) caused by the integrated mutant *cmd1* gene.

"Low dose" means that the mutant *cmd1* gene, again driven by its normal promoter, was present on a centromere-containing plasmid. As shown in Figure 1B, the mutant *cmd1* gene was placed on a centromere vector marked with *TRP1* and introduced into a different strain [YOC103; *MAT***a** *ade2 lys2 his3 trp1 leu2 ura3 cmd1-Δ1::HIS3* (pRB1612)] which again contains *cmd1-Δ1* and the wild-type *CMD1* on a *URA3* plasmid. Loss of the *URA3-CMD1* plasmid was again selected using FOA plates. We reasoned that the copy number of a centromere vector is 1–3 (possibly higher under selection), thus a strain with *cmd1* on a *CEN* plasmid should display any mutant phenotype in a "low dose" condition.

"High dose" means that the mutant calmodulin sequence was introduced (exactly as before) on a centromere plasmid, but this time driven by the galactose-inducible *GAL1* promoter. The phenotype was assessed under conditions where galactose is the sole carbon

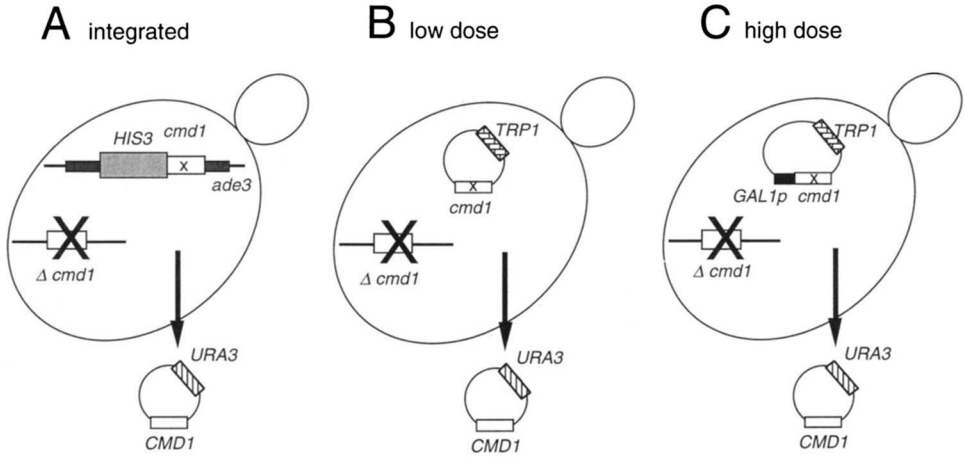


FIGURE 1.—Schematic diagram representing three strategies for testing phenotypes of the yeast calmodulin mutants. The Phe → Ala *cmd1* mutations were integrated at the *ade3* locus of the chromosome (A, integrated), or placed on a centromere plasmid (B, low dose; and C, high dose) and under the *GAL1* promoter (C, high dose). Calmodulin was expressed under its own promoter (A and B) or by the *GAL1* promoter (C). The mutant phenotype was examined after elimination of the wild-type *CMD1* plasmid marked with *URA3* (pRB1612) on FOA plates.

source. Under these circumstances, we found previously an expression level of calmodulin about 20-fold higher than the normal level (OHYA and ANRAKU 1989b).

**A single Phe to Ala mutant with a temperature-sensitive phenotype:** We began characterization of the new *cmd1* mutants by examining the ability to complement *cmd1-Δ1::TRP1* after integration of the mutations into the chromosome (Figure 1A). All of the strains that harbored the Phe → Ala mutations (*cmd1-221–cmd1-227*) could form colonies on FOA plates at 25°, indicating that none of the mutations is lethal. Single colonies purified from these FOA plates were then tested across a broad temperature range (14–37.5°). As summarized in Figure 2, we found that all mutants save *cmd1-226* (F92A) grew as well as the wild-type control strain at all temperatures. The *cmd1-226* mutant did not grow very well on rich (YPD) agar plates above 35.5°; it also showed a weak cold-sensitive phenotype (Figure 3). The growth rates of the mutants were measured; every mutant that showed robust growth on plates grew in liquid medium (YPD) at rates essentially equivalent to the wild-type strain both at 25° (2.1–2.3-hr doubling time) and 37° (1.7–1.8-hr doubling time). The *cmd1-226* mutant grew slowly (3.5-hr doubling time) even at 25°. These results indicate that all but one (F92A) of the Phe → Ala mutants, unlike a deletion (DAVIS *et al.* 1986), have no obvious deleterious effect.

When the *cmd1-226* (F92A) mutation was introduced on a centromere plasmid (*i.e.*, low dose condition), its growth profile was noticeably different. It now grew well below 35.5°, grew slowly at 37° and formed very tiny colonies even at 37.5°. Thus under the low dose condition, only a partial temperature-sensitive phenotype could be observed (Figure 3). Introduction of *cmd1-226* on a Gal-promoter plasmid (*i.e.*, high dose condition) suppressed its temperature-sensitive growth phenotype to the same degree (Figure 3), suggesting that a modest

| Mutants | Position | Phenotype |
|---|---|---|
| | -F12--F16-F19----F65-F68---F89-F92--F140 | |
| *cmd1-221* | -A---------- | "Wild-type" |
| *cmd1-222* | ------A---A---------- | "Wild-type" |
| *cmd1-223* | ----------A---------- | "Wild-type" |
| *cmd1-224* | ----------A---------- | "Wild-type" |
| *cmd1-225* | ----------A---------- | "Wild-type" |
| *cmd1-226* | ----------A---------- | Ts |
| *cmd1-227* | ----------A- | "Wild-type" |

FIGURE 2.—Growth of the Phe → Ala *cmd1* mutant strains. "Wild-type" means no discernible growth defect. Independently isolated mutant strains in a MATα background (YOC321, YOC322, YOC323, YOC324, YOC325, YOC326 and YOC327; see Table 2) gave the same results.

overproduction of the mutant protein suffices for growth at all but the highest temperatures.

**Most double Phe → Ala mutants showed either temperature-sensitive (Ts) or lethal phenotype:** Despite our success in finding, for the first time, a single *cmd1* mutation with a phenotype, we were still puzzled by the failure of so many of the others to show phenotypes. Reasoning that there might be internal structural redundancy in the molecule, we systematically constructed multiple Phe → Ala mutants. Alleles *cmd1-229* to *cmd1-233* and *cmd1-239* to *cmd1-248* are Phe → Ala double mutations, and *cmd1-228* and *cmd1-234* to *cmd1-238* contain F16A, F19A (which we are treating as a single lesion) and one additional Phe → Ala mutation. Alleles, *cmd1-249* to *cmd1-252* contained multiple lesions within the N-terminal lobe so that only one Phe residue (two in the case of F16 F19) remained in the N-terminal half domain. All of the phe residues in the N-terminal half domain were changed to Ala in *cmd1-253*.

Figure 4 shows that 11 out of 26 strains that harbored the integrated mutations did not form colonies well on FOA plates at 25° (see MATERIALS AND METHODS). We checked the plasmid loss phenotype using FOA plates at

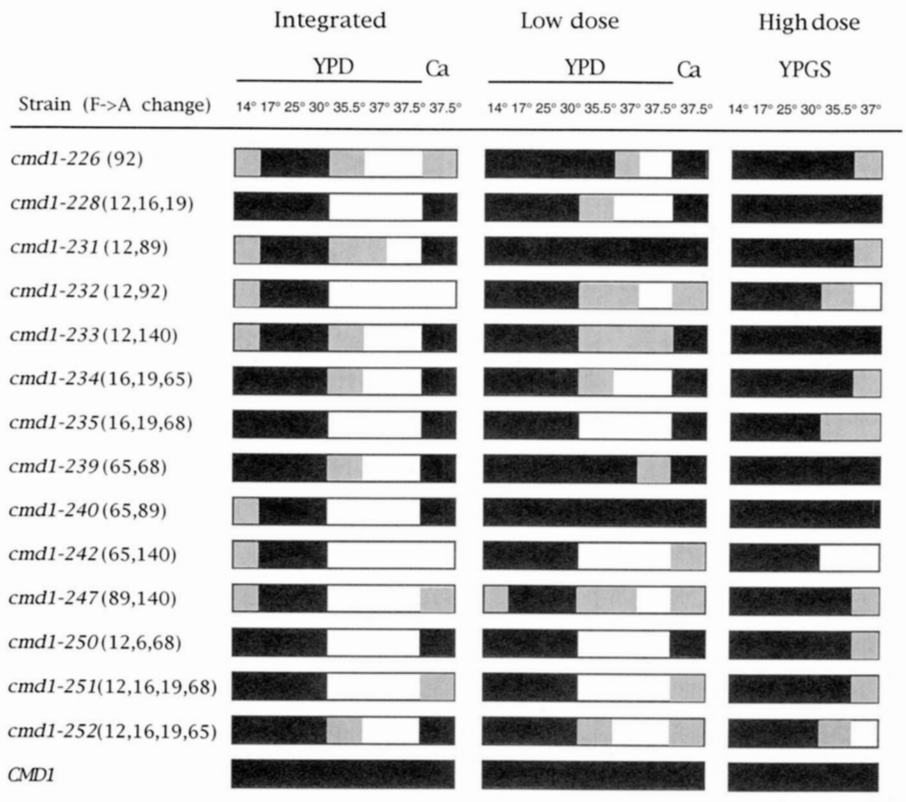


FIGURE 3.—Growth of the Phe → Ala *cmd1* mutants assessed in the "integrated," "low dose" and "high dose" condition. Growth at each temperature was scored on YPD agar plates, YPGS agar plates and YPD agar plates containing 100 mM $CaCl_2$ (Ca), and is relative to growth of the wild-type strain at the same temperature. Black indicates growth equivalent to wild type, gray indicates weak growth, and white indicates no growth.

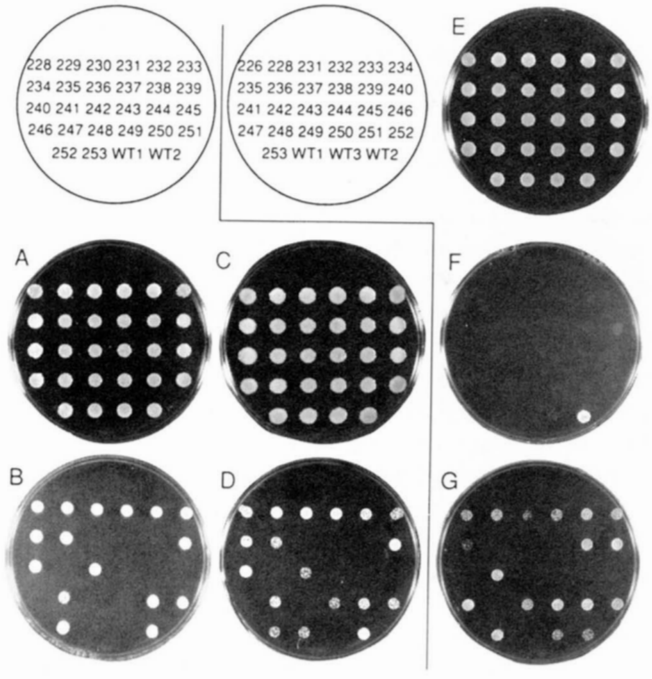


FIGURE 4.—Examination of lethal phenotype caused by the Phe → Ala *cmd1* mutations. Integration (A, B), low dose (C, D) and high dose (E, F, G) were used to check loss of the wild-type *CMD1* plasmid (see Figure 1 and MATERIAL AND METHODS for detail). Permissive agar plates (YPD; A, C and YPGS; E) and FOA agar plates (FOA-Glc; B, D, F and FOA-GS; G) were incubated at 25°. Wild-type control strains were YOC101 (WT1), YOC200 (WT2) and YOC500 (WT3).

17° and 30° as well, with essentially the same results. This indicates that these eleven *cmd1* mutations cause lethality on synthetic medium.

We then examined the growth phenotypes of these 11 mutants on rich medium. Heterozygous *CMD1/cmd1* diploid strains were constructed, sporulated and dissected. Table 3 shows the results of tetrad analysis. When dissected on YPD plates at 25°, all the heterozygous diploids produced a maximum of two viable spores. However, when dissected on YPD plates containing 100 mM $CaCl_2$, tetrads heterozygous for *cmd1-236*, *cmd1-241*, *cmd1-245*, *cmd1-246*, *cmd1-249* and *cmd1-253* often produced three or four viable spores. White colonies were found among the spores in these crosses, showing that the *ade3::cmd1:HIS3* alleles were retained in these segregants. These strains do not contain, however, an intact *CMD1* gene: the *cmd1* segregants did not grow well on synthetic medium supplemented with the nutrients required because of the markers. This is consistent with the previous observation (Figure 4) that strains harboring these mutations with *CMD1* on a *URA3* plasmid did not form colonies on synthetic FOA plates. Although addition of 100 mM $CaCl_2$ was necessary for these *cmd1* mutants to grow into colonies from spores (Table 4), they did not require 100 mM $CaCl_2$ for growth after making colonies. All of them failed grow on rich medium at 37°, showing a temperature-sensitive phenotype. Based on these results, we concluded that *cmd1-236*, *cmd1-241*, *cmd1-245*, *cmd1-246*, *cmd1-249* and *cmd1-253* cause a lethal phenotype on synthetic medium, but a conditional-lethal phenotype on rich

**TABLE 3**

**Tetrad analysis of diploid strains heterozygous for the Phe→Ala *cmd1* mutations**

| | YPD | | | | | YPD+100 mM $CaCl_2$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *CMD1* allele (F→A change) | 4+:0− | 3+:1− | 2+:2− | 1+:3− | 0+:4− | 4+:0− | 3+:1− | 2+:2− | 1+:3− | 0+:4− |
| *cmd1-236* (16 19 89) | 0 | 0 | 21 | 4 | 0 | 8 | 7 | 5 | 2 | 0 |
| *cmd1-237* (16 19 92) | 0 | 0 | 14 | 9 | 0 | 0 | 0 | 16 | 7 | 0 |
| *cmd1-238* (16 19 140) | 0 | 0 | 20 | 0 | 3 | 0 | 0 | 23 | 0 | 0 |
| *cmd1-241* (65 92) | 0 | 0 | 10 | 11 | 1 | 11 | 5 | 4 | 0 | 0 |
| *cmd1-243* (68 89) | 0 | 0 | 19 | 4 | 0 | 0 | 0 | 21 | 1 | 2 |
| *cmd1-244* (68 92) | 0 | 0 | 16 | 8 | 0 | 0 | 0 | 15 | 7 | 0 |
| *cmd1-245* (68 140) | 0 | 0 | 22 | 3 | 0 | 23 | 0 | 5 | 2 | 0 |
| *cmd1-246* (89 92) | 0 | 0 | 15 | 7 | 0 | 16 | 1 | 4 | 0 | 0 |
| *cmd1-248* (92 140) | 0 | 0 | 15 | 2 | 5 | 0 | 0 | 25 | 0 | 0 |
| *cmd1-249* (16 19 65 68) | 0 | 0 | 14 | 5 | 5 | 2 | 11 | 11 | 2 | 0 |
| *cmd1-253* (12 16 19 65 68) | 0 | 0 | 20 | 2 | 2 | 0 | 7 | 15 | 0 | 2 |
| Wild type | 21 | 4 | 0 | 0 | 0 | 23 | 0 | 0 | 0 | 0 |

Haploid *MAT***a** strains harboring integrated *cmd1* mutations and pRB1612 were crossed with YOC300. After eliminating pRB1612 on FOA plates, the diploids were sporulated and subjected to tetrad analysis. Tetrad dissection was performed either on the YPD palates or the YPD plates containing 100 mM $CaCl_2$. Number of viable (+) and inviable (−) spores was scored in each ascus.

**TABLE 4**

**Steady-state level and electrophoretic mobilities of calmodulin in the lethal *cmd1* mutants and wild-type (WT) controls**

| Mutations (F→A change) | Relative amount of calmodulin | Apparent $M_r$ (kDa) | |
|---|---|---|---|
| | | $Ca^{2+}$ | EGTA |
| *cmd1-236* (16 19 89) | 1.0 | 13.7 | 16.5 |
| *cmd1-237* (16 19 92) | 1.2 | 13.3 | 16.5 |
| *cmd1-238* (16 19 140) | 0.9 | 13.3 | 16.9 |
| *cmd1-241* (65 92) | 1.5 | 13.3 | 16.9 |
| *cmd1-243* (68 89) | 1.6 | 13.7 | 16.1 |
| *cmd1-244* (68 92) | 1.0 | 13.3 | 16.1 |
| *cmd1-245* (68 140) | 1.7 | 13.0 | 16.1 |
| *cmd1-246* (89 92) | 1.6 | 13.3 | 16.5 |
| *cmd1-248* (92 140) | 0.4 | 13.3 | 16.1 |
| *cmd1-249* (16 19 65 68) | 0.7 | 14.3 | 17.3 |
| *cmd1-253* (12 16 19 65 68) | 0.6 | 15.6 | 17.7 |
| Wild type [YPH499] | 1.0 | 13.0 | 15.4 |
| Wild type [YOC200] | 1.0 | 13.0 | 15.4 |

The lethal calmodulin strains were maintained with pRB1619 expressing chicken calmodulin under the *GAL1* promoter. Mutant and wild-type calmodulins were analyzed by immunoprecipitation, after cells were labeled with [$^{35}$S]$SO_4$ for 30 min as described in MATERIALS AND METHODS. Actin was used as an internal control and data are represented as relative values to the wild-type control (YPH499). Apparent $M_r$ of mutant calmodulin was calculated using carbonic anhydrase, trypsin inhibitor and lysozyme as molecular markers.

medium. The remaining mutations, *cmd1-237*, *cmd1-238*, *cmd1-243*, *cmd1-244*, *cmd1-248*, were lethal on both synthetic and rich media.

We analyzed the non-lethal mutant strains in terms of the growth phenotypes across a broad temperature range (14–37.5°), and found that almost all of the "double" and all "heavily mutagenized" integrated Phe → Ala mutations did not grow well at 37.5° (Figure 3). Exceptions were the *cmd1-229* (F12A F65A) and *cmd1-230* (F12A F68A) mutant strains, which grew as well as the wild-type controls at any temperature. Some mutant strains (*cmd1–331*, *cmd1-232*, *cmd1-233*, *cmd1-240*, *cmd1-242*, and *cmd1-247*) grew slowly at 14°, but did not show strong cold-sensitive phenotypes. Variation of the phenotype was seen at intermediate temperature (Figure 3). At 35.5°, some mutants (*cmd1-228*, *cmd1-232*, *cmd1-235*, *cmd1-240*, *cmd1-242*, *cmd1-247*, *cmd1-250* and *cmd1-251*) did not grow, whereas others (*cmd1-231*, *cmd1-233*, *cmd1-234*, *cmd1-239* and *cmd1-252*) grew slowly.

**Effect of overproduction:** When the "double" and "heavily-mutagenized" mutations were placed either on a centromere plasmid ("low dose") or under the *GAL1* promoter ("high dose"), the phenotypic consequences were markedly different. Some Ts$^-$ mutations (*cmd1-231*, *cmd1-233*, *cmd1-239* and *cmd1-240*), when placed on a centromere plasmid, allowed cell growth even at the most restrictive temperature (37.5°). Furthermore, two of the Ts$^-$ mutants (*cmd1-231* and *cmd1-240*), at high temperature in a low dose condition, began to grow as well as wild-type (Figure 3). When grossly overexpressed, other mutations (*cmd1-228*, *cmd1-232*, *cmd1-234*, *cmd1-235*, *cmd1-247*, *cmd1-250*, *cmd1-251*) also began to allow growth at previously non-permissive temperature. Other alleles (*cmd1-242* and *cmd1-252*) still resulted in a Ts$^-$ phenotype even when overexpressed by the *GAL1* promoter (Figure 3).

Figure 5 summarizes the growth phenotypes of the double and heavily-mutagenized Phe → Ala mutants. Of the 26 Phe → Ala mutants analyzed, 5 were dose-independent recessive lethal, 6 were lethal only in synthetic medium, 2 were dose-independent temperature-sensitive, 11 were dose-suppressible temperature-sensitive, and 2 had no discernible phenotype.

**Protein stability of mutant calmodulin:** One possible explanation for the recessive-lethal phenotypes might be that the mutant calmodulin becomes unstable. In order to test this possibility, we measured the steady-state expression levels of calmodulin in cells expressing each of the 11 mutants lethal in synthetic and/or rich medium described above. The design of these studies takes advantage of two traits of vertebrate calmodulin: expression of vertebrate calmodulin complements the yeast *cmd1* deletion mutation (and, as it turns out, the new

| Mutant | Position -F12--F16-F19----F65-F68---F89-F92--F140 | Phenotype | High dose suppression |
|---|---|---|---|
| A) Double mutants | | | |
| *cmd1-228* | -A----A---A---------- | Ts | Yes |
| *cmd1-229* | -A---------------A---------- | "Wild-type" | |
| *cmd1-230* | -A-----------------A---------- | "Wild-type | |
| *cmd1-231* | -A-------------------------A---------- | Ts | Yes |
| *cmd1-232* | -A-----------------------------A------- | Ts | Yes |
| *cmd1-233* | -A-----------------------------------A- | Ts | Yes |
| *cmd1-234* | ------A---A------A---------- | Ts | Yes |
| *cmd1-235* | ------A---A----------A ---------- | Ts | Yes |
| *cmd1-236* | ------A---A----------------A---------- | lethal(S) | No |
| *cmd1-237* | ------A---A--------------------A------- | lethal | No |
| *cmd1-238* | ------A---A---------------------------A- | lethal | No |
| *cmd1-239* | ----------------A---A---------- | Ts | Yes |
| *cmd1-240* | ----------------A---------A---------- | Ts | Yes |
| *cmd1-241* | ----------------A-------------A------- | lethal(S) | No |
| *cmd1-242* | ----------------A------------------A- | Ts | No |
| *cmd1-243* | --------------------A-----A---------- | lethal | No |
| *cmd1-244* | --------------------A---------A------- | lethal | No |
| *cmd1-245* | --------------------A---------------A- | lethal(S) | No |
| *cmd1-246* | -------------------------A---A------- | lethal(S) | No |
| *cmd1-247* | -------------------------A---------A- | Ts | Yes |
| *cmd1-248* | -------------------------------A-----A- | lethal | No |
| B) Heavily-mutagenized mutants | | | |
| *cmd1-249* | ------A---A------A---A---------- | lethal(S) | Yes |
| *cmd1-250* | -A---------------A---A---------- | Ts | Yes |
| *cmd1-251* | -A----A---A----------A---------- | Ts | Yes |
| *cmd1-252* | -A----A---A------A---------- | Ts | No |
| *cmd1-253* | -A----A---A------A---A---------- | lethal(S) | Yes |

FIGURE 5.—Summary of the growth properties of the double (A) and multiple (B) Phe → Ala *cmd1* mutant strains. Phenotypes of the single-copy integrated mutant strains are shown. High dose suppression includes any phenotypic suppression either under low dose and high dose condition.

lethal mutations) and antibody raised against yeast calmodulin does not cross-react with vertebrate calmodulin (OHYA and ANRAKU 1989a). In this experiment, we used strains containing the *cmd1* deletion, the integrated lethal *cmd1* mutations at the *ade3* locus and a plasmid with the chicken calmodulin gene expressed under the *GAL1* promoter. Absence of a wild-type yeast calmodulin gene in these strains was tested by checking for inability to grow on glucose plates, where the vertebrate calmodulin is not expressed.

Production of wild-type and the mutant calmodulin was quantified by immunoprecipitation using polyclonal antibodies raised against yeast calmodulin (Table 4). Single copy *CMD1* integrated at the *ade3* locus did not affect the calmodulin expression level, since the wild-type strain (YPH499) and the *ade3::CMD1* integrated strain (YOC200) had equivalent levels of calmodulin. Many of the lethal mutants proved to contain essentially wild-type levels of calmodulin (*cmd1-236*, *cmd1-237*, *cmd1-238* and *cmd1-244*) or levels slightly higher that wild type (*cmd1-241*, *cmd1-243*, *cmd1-245* and *cmd1-246*). There were only three mutant strains (*cmd1-248*, *cmd1-249* and *cmd1-253*) that contained obviously lower levels of calmodulin.

Protein stability was then examined more directly by pulse-chase labeling of representative strains (*CMD1* control, *cmd1-241* and *cmd1-253*, each integrated at *ade3*) followed by immunoprecipitation. After 3 hr incubation at 30°, 80 and 70% of the calmodulin still remained in the wild-type and *cmd1-241* cells, respectively. Even in the *cmd1-253* cells, which contain a lower steady-state level of calmodulin (Table 4), only 50% of the calmodulin had been degraded in 3 hr (Figure 6). Taken together, these data show that most of the lethal Phe → Ala mutant calmodulins are as stable as wild-type calmodulin. For these mutants the lethal phenotype must be due to differences in the quality of the protein, and not to any gross differences in amount.

**$Ca^{2+}$-suppressible phenotypes:** Mutations in a number of yeast $Ca^{2+}$-binding proteins, including calmodulin, protein kinase C (Pkc1), the β-subunit of geranylgeranyl transferase I (Cal1p/Cdc43p) confer $Ca^{2+}$-suppressible phenotypes (ANRAKU *et al.* 1991; OHYA and ANRAKU 1992; MAYER *et al.* 1992). We found that the Phe → Ala calmodulin mutants described here also possess this property. Addition of 100 mM $CaCl_2$ to the medium suppressed, to various extents, the $Ts^-$ phenotypes of most integrated $Ts^-$ mutants (Figure 3). There were a few exceptions: *cmd1-232* and *cmd1-242*. These exceptional mutants were suppressed, but only to a very limited extent, by 100 mM $CaCl_2$ in the "low dose" condition (Figure 3).

Like other calmodulins, mobility of yeast calmodulin

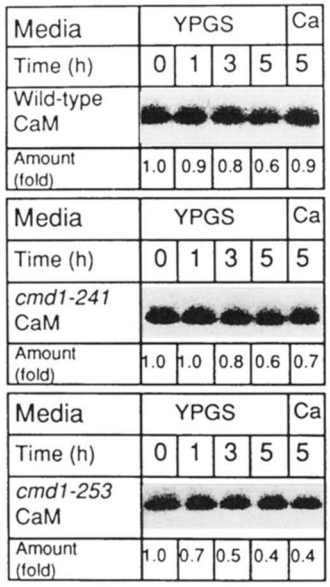


FIGURE 6.—Stability of calmodulin in the wild-type and two lethal *cmd1* (*cmd1-241* or *cmd1-253*) mutants assessed by a pulse-chase experiment. Haploid strains bearing pRB1619 (chicken calmodulin) and integrated *CMD1*, *cmd1-241* or *cmd1-253* were grown in galactose-containing medium and subjected to the pulse-chase protocol described in the Methods. The ratio of calmodulin at each indicated time point relative to the calmodulin at time zero is given.

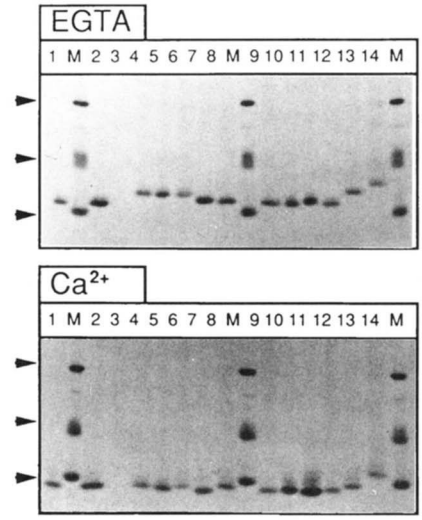


FIGURE 7.—$Ca^{2+}$-dependent electrophoretic mobility shifts of the wild-type calmodulin and the mutant calmodulins. Immunoprecipitated calmodulin in the wild-type control and the lethal *cmd1* mutants were analyzed as described in MATERIALS AND METHODS. Wild-type (1 and 2), *cmd1-236* (4), *cmd1-237* (lane 5), *cmd1-238* (lane 6), *cmd1-241* (lane 7), *cmd1-243* (lane 8), *cmd1-244* (lane 9), *cmd1-245* (lane 10), *cmd1-246* (lane 11), *cmd1-248* (lane 12), *cmd1-249* (lane 13), *cmd1-253* (lane 14) calmodulin were analyzed. Lane 3 contains the immunoprecipitates from the cells bearing the *cmd1* deletion (*cmd1-Δ1::TRP1*) and pRB1619 (chicken calmodulin) as a control to show that the anti-yeast antibody does not cross-react with the chicken calmodulin. M indicates molecular weight markers (Carbonic anhydrase, Trypsin inhibitor and Lysozyme). Apparent $M_r$ of the mutant calmodulins is listed in Table 4.

in an SDS polyacrylamide gel is shifted in a $Ca^{2+}$-dependent manner (WATTERSON *et al.* 1980; OHYA and ANRAKU 1989a), presumably because calcium binding causes a conformational change. All the recessive lethal Phe → Ala mutant calmodulin showed very similar $Ca^{2+}$-dependent mobility shifts (Figure 7, Table 4), indicating that all of the mutants retained their $Ca^{2+}$-binding activity. There are characteristic differences in mobility among the mutants, especially in the absence of $Ca^{2+}$, the significance of which is not understood.

**Intragenic complementation of the temperature-sensitive mutations:** Recently we reported intragenic complementation among the calmodulin mutants (OHYA and BOTSTEIN 1994). Here we present complementation data for reciprocal matings of 14 different *cmd1-ts* alleles (Table 5). All diploid strains homozygous for the temperature-sensitive *cmd1* alleles did not grow at the restrictive temperature, but diploids bearing different *cmd1* alleles often were able to grow. Heteroallelic diploids constructed with *cmd1-231* or *cmd1-233* grew relatively slowly, or even did not grow in one combination (*cmd1-232/cmd1-233*) because of the partial dominance of these alleles. All the heteroallelic diploids constructed with *cmd1-242* or *cmd1-247* grew very badly at restrictive temperature; this is likely to be due to dominance, as each of these mutations showed partial dominance when mated with *CMD1* strains. Analyses of all combinations of diploids harboring recessive alleles revealed four intragenic complementation groups (A–D). Since *cmd1-234*, *cmd1-235*, *cmd1-240*, *cmd1-251* and *cmd1-252* did not complement mutations in more than one of the four groups, we suppose that these alleles harbor multiple or overlapping functional defects.

## DISCUSSION

The mutants described above were isolated on the basis of an inference, from structural data, that the highly conserved phenylalanine residues play an important role in the interaction of calmodulin and target proteins. The success of this approach, after so much difficulty in recovering calmodulin with phenotypes, can be seen as support for this inference.

Random mutagenesis of yeast calmodulin *in vitro* previously had yielded unexpectedly low numbers of the conditional and lethal mutations. In addition to the possibility of the dosage artifacts the we documented for some of the mutants, this low yield could be explained most easily in two ways: the essential region of the protein that is vulnerable to mutation is very limited in size (comprising a small percentage of the total structure) or the essential region of the protein is duplicated, resulting in functional redundancy. Our results point toward the latter explanation. We found that all but one of the single Phe → Ala mutations still cause no obvious growth

**TABLE 5**

**Intragenic complementation among the *cmd1-ts* mutations**

| | *MATα* strain | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MAT***a** strain (group: F→A change) | *cmd1-226* (A) | *cmd1-232* (A) | *cmd1-228* (B) | *cmd1-231* (C) | *cmd1-233* (C) | *cmd1-239* (D) | *cmd1-250* (D) | *cmd1-234* | *cmd1-235* | *cmd1-240* | *cmd1-251* | *cmd1-252* | *cmd1-242* | *cmd1-247* | *CMD1* |
| *cmd1-226* (A: 92) | – | – | + | ± | ± | + | + | + | + | + | + | + | – | – | + |
| *cmd1-232* (A: 12 92) | – | – | + | ± | – | + | + | ± | ± | + | + | ± | – | – | + |
| *cmd1-228* (B: 12 16 19) | + | + | – | + | ± | + | + | – | – | – | – | – | – | – | + |
| *cmd1-231* (C: 12 89) | ± | ± | + | – | – | + | ± | – | ± | ± | ± | – | – | – | + |
| *cmd1-233* (C: 12 140) | ± | – | ± | – | – | ± | ± | – | – | – | – | – | – | – | + |
| *cmd1-239* (D: 65 68) | + | + | + | + | ± | – | – | – | – | – | – | – | – | – | + |
| *cmd1-250* (D: 12 65 68) | + | + | + | + | ± | – | – | – | – | – | – | – | – | – | + |
| *cmd1-234* (16 19 65) | + | + | – | – | – | – | – | – | – | – | – | – | – | – | + |
| *cmd1-235* (16 19 68) | + | + | – | ± | – | – | – | – | – | – | – | – | – | – | + |
| *cmd1-240* (65 89) | + | + | – | – | – | – | – | – | – | – | – | – | – | – | + |
| *cmd1-251* (12 16 19 68) | ± | ± | – | ± | – | – | – | – | – | – | – | – | – | – | + |
| *cmd1-252* (12 16 19 65) | ± | ± | – | – | – | – | – | – | – | – | – | – | – | – | + |
| *cmd1-242* (65 140) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | ± |
| *cmd1-247* (89 140) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | ± |
| *CMD1* | + | + | + | + | + | + | + | + | + | + | + | + | ± | ± | + |

*MAT***a** and *MATα* strains, all of which were *leu2 lys2*, were transformed with pRS315 (*LEU2*) and pRS317 (*LYS2*) (SIKORSKY and HIETER 1989), respectively in order to provide complementary auxotrophies for selection of diploids. Diploids were selected and growth was examined on YPD medium at 25° and 37.5°. Growth at the high temperature is indicated as follows: +, robust growth; ±, less vigorous growth; –, no growth.

phenotype. However, a large fraction of the double mutations turned out to be either lethal or to cause a readily scored temperature-sensitive phenotype.

Most of the lethal mutants contained levels of mutant calmodulin equivalent to wild-type calmodulin. This means that the mutant phenotypes are not due to instability of mutant calmodulins, but instead are due to impairment of calmodulin function(s). We can now suggest further that the phenylalanine residues are important for essential function(s) of calmodulin, but they are nevertheless functionally redundant.

Functional redundancy has to be seen also in the context of the diversity of essential functions carried out by calmodulin in yeast (OHYA and BOTSTEIN 1994) (see also below). The overall picture that emerges is that the several phenylalanine residues in calmodulin are required to different extents in different combinations in order to carry out each of the several essential tasks. In each of these tasks, there is a different degree to which each of the symmetrical lobes can compensate for the other.

**Dose-dependent phenotypes:** In characterizing the phenotype of the calmodulin mutants, we paid considerable attention to the copy number of calmodulin. We hypothesized that calmodulin mutants with low affinity to targets might be overcome stoichiometrically by increasing the amount of mutant calmodulin. Therefore, for the most rigorous test of mutant phenotype, we expressed the mutant protein under a situation (integration into a known locus) that virtually guarantees a single copy condition. Using the three dosage levels, we were able to identify a series of single and multiple Phe → Ala calmodulin mutations showing both dose-suppressible and dose-independent deleterious phenotypes.

It is worth emphasizing that many of our mutants lost most or all of the phenotype that could be observed readily in single-copy when placed on a CEN plasmid. This means that CEN plasmids cannot be thought of as equivalent to single-copy when analyzing function of gene products.

**Ranking the significance of the phenylalanine residues:** Detailed analysis of the double mutants indicated that the many phenylalanine residues do not contribute equally to function. Different combinations of the mutated sites resulted in three phenotypes: "Wild-type," $Ts^-$, and lethal. We can derive a hierarchy by ranking the phe residues based on the frequency with which they contribute to the more severe phenotypes. This method results in the order: F92 > F89 = F140 = F16F19 > F68 > F65 > F12, where F92 is the most important phe residue. This ranking is gratifyingly consistent with the observation that F92 is the only position that causes a growth phenotype as a single Phe → Ala mutant. By this ranking scheme, the three C-terminal phe residues appear to be more crucial for calmodulin function than the N-terminal Phe residues.

The significance of the phe residues can be assessed in another way: by ranking the relative severity of the phenotypes of mutants with only one intact phe residue in either the N-terminal lobe (*cmd1-249, cmd1-250, cmd1-251, cmd1-252*) or C-terminal lobe (*cmd1-248, cmd1-247, cmd1-246*). This scheme gives F16F19 = F68 = F65 > F12 in the N-terminal lobe and F92 > F89 = F140 in the C-terminal lobe. This assessment is consistent with the first ranking method.

**Interactions among the phenylalanine residues:** Figure 8 illustrates graphically the genetic interactions among the phe residues in calmodulin. In total we tested

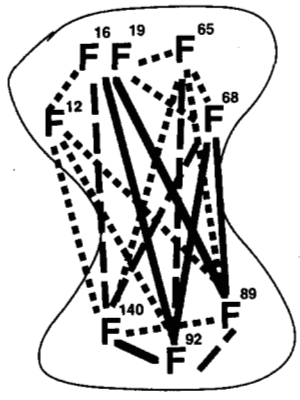


FIGURE 8.—Functional interaction among phenylalanine residues in calmodulin sequence. When double mutants are lethal the residues are connected with a solid line; when they are lethal in synthetic medium only, a broken line; when they are temperature-sensitive, a dotted line. The N-terminal lobe is at the top and the C-terminal lobe is at the bottom of the figure.

21 combinations and found that 19 have phenotypes more severe than either single lesion: 5 combinations produce a fully lethal phenotype, 4 produce a lethal phenotype on minimal medium, and 10 produce a temperature-sensitive phenotype.

These genetic interactions must be interpreted in light of the three dimensional structure of calmodulin (BABU *et al.* 1988; IKURA *et al.* 1992; MEADOR *et al.* 1992). Clustering of the phe residues in each domain (BABU *et al.* 1988) helps to explain synthetic interactions that are observed within the same half domain of calmodulin. Simultaneous alteration of the phe residues likely decreases disproportionately the surface hydrophobicity of the calmodulin lobes, weakening the hydrophobic interaction between the lobe and the target proteins.

Strong inter-domain synthetic interaction among our mutations is observed, as might be expected on the basis of our hypothesis of functional redundancy. For example, F68A in the N-terminal half domain has lethal interactions with two of the three Phe → Ala mutations in the C-terminal lobe, whereas it has either $Ts^-$ or no synthetic interaction with other mutations in the N-terminal lobe. The interactions between N-terminal lobe and C-terminal lobe can be understood in light of the three dimensional model of calmodulin-calmodulin binding peptide complex (IKURA *et al.* 1992; MEADOR *et al.* 1992). Although direct interaction between the two lobes was not observed in the absence of the target peptides, in the calmodulin-calmodulin-binding peptide complex, the target peptide is engulfed by both lobes in a manner akin to an articulated jaw; cooperatively by the two lobes.

Cooperativity in calmodulin action has been often demonstrated in biochemical analysis (YAZAWA *et al.* 1990; COHEN and KLEE 1988). Although either half-calmodulin fragment possesses ability to bind target proteins and to activate several calmodulin target enzymes, more efficient binding and activation is observed with intact calmodulin.

**Comparison between two half domains:** It was unexpected that the C-terminal domain mutation, *cmd1-248* (F92A F140A) caused a lethal phenotype even when overproduced, since SUN *et al.* (1991) have shown that overexpression of either half-calmodulin is sufficient for cell growth. Our interpretation is that overexpression of the N-terminal half is sufficient for cell growth through substitution for the C-terminal half, but that association of the inactive C-terminal half-calmodulin somehow compromises the effect. A simple competition model is complicated by the observation that the most severe N-terminal mutant *cmd1-253*, which has all five phe residues in the N-terminal half altered, caused a dose-suppressible lethal phenotype similar to the half-calmodulin mutants.

We often observed increased severity of phenotype in the C-terminal mutants over the N-terminal mutants (Figure 8). This cannot be explained simply by a difference in calmodulin levels. N-terminal mutant calmodulin (*cmd1-253*) is even less stable than the C-terminal mutant calmodulin (*cmd1-246* and *cmd1-248*). For this we offer two suggestions. First, the essential function of the N-terminal lobe may be less important than that of the C-terminal lobe. Functional differences between the two lobes have been suggested for Paramecium calmodulin mutants that show behavioral phenotypes (HINRICHSEN *et al.* 1991; KUNG *et al.* 1992) and also in biochemical analysis of half-calmodulin *in vitro* (COHEN and KLEE 1988). In support of this idea we also have observed that when integrated into the chromosome, expression of the N-terminal half-calmodulin under the *GAL1* promoter does not support cell growth while the C-terminal half-calmodulin does (Y. OHYA and D. BOTSTEIN, unpublished results). Second, there may be amino acid residues other than phe that are functionally redundant with the N-terminal phe residues. The three dimensional structure of calmodulin-calmodulin binding peptide complex demonstrates that other amino acid residues such as methionine are also involved in interaction with calmodulin-calmodulin target peptide IKURA *et al.* 1992). This notion could be tested by combining mutations in these residues with the Phe → Ala mutation of the N-terminal domain.

**Complementation among *cmd1-ts* mutations:** Genetic analyses of 14 temperature-sensitive calmodulin mutations revealed four intragenic complementation groups. It should be noted that diverse phenotypes were observed with typical mutants of each group: *cmd1-226*, *cmd1-228*, *cmd1-233* and *cmd1-239* showed different characteristic functional defects in actin organization, calmodulin localization, nuclear division and bud emergence, respectively (OHYA and BOTSTEIN 1994). Analysis of the level of calmodulin protein and stability of the

protein makes it unlikely that the difference is due simply to differing levels of protein (Table 4, Figure 7). Therefore, the most likely supposition is that calmodulin mutants belonging to different complementation group have defects in interaction with different target proteins, each corresponding to the essential calmodulin functions. As for the calmodulin mutations which do not complement with mutations of two or more complementation groups, we suppose that these alleles harbor multiple or overlapping functional defects. However, we cannot exclude the possibility that these mutant calmodulins are perturbed in overall structure.

Recently, two essential targets of calmodulin were found in yeast: Myo2p (BROCKERHOFF *et al.* 1994) and Nuf1p/Spc110 (GEISER *et al.* 1993). Myo2p, a myosin homolog with calmodulin binding sites, is involved in bud emergence (JOHNSTON *et al.* 1991). Recently it was shown biochemically that Myo2p indeed binds calmodulin (BROCKERHOFF *et al.* 1994). Given the phenotypic similarity between the *cmd1-233* and *myo2-66* mutants (JOHNSTON *et al.* 1991; OHYA and BOTSTEIN 1994), the calmodulin made from the *cmd1-233* mutant gene may be unable to interact with Myo2p, thereby causing a budding defect. Nuf1p, a coiled-coil protein with a calmodulin-binding site (MIRZAYAN *et al.* 1992; GEISER *et al.* 1993), likely plays important functions of spindle pole body (SPB), since it is located within it (KILMARTIN *et al.* 1993). Furthermore, *NUF1* was identified not only as a gene resulting in dominant suppression of *cmd1-1* but also as a gene encoding a protein capable of binding calmodulin (GEISER *et al.* 1993). *cmd1-239* causes characteristic defects in SPB function (OHYA and BOTSTEIN 1994), consistent with the view that *cmd1-239* calmodulin cannot bind Nuf1p efficiently.

**$Ca^{2+}$-suppressible phenotypes of the calmodulin mutants:** GEISER *et al.* (1991) proposed that calmodulin performs its essential function without binding $Ca^{2+}$. This is based on the observation that their mutant calmodulin that has no apparent ability to bind $Ca^{2+}$ *in vitro* still supports the yeast growth well. We observed that many Phe → Ala mutant calmodulins that retain $Ca^{2+}$-binding ability result in $Ca^{2+}$-suppressible phenotype, suggesting that $Ca^{2+}$-binding activity may be important for function, at least in the mutant condition. Supplementation with high concentration of $CaCl_2$ was also required for growth of several *cmd1* mutants from spores (Table 3). Similar $Ca^{2+}$-suppressible phenotypes have been also seen with the half-calmodulin mutants (SUN *et al.* 1991).

To account for these results, one can consider several explanations. One of these, $Ca^{2+}$-induced stabilization of the protein, is ruled out by our observations of the stability of the protein *in vivo*. However, a $Ca^{2+}$-induced stabilization of the protein *conformation* is still possible. One would hypothesize that in wild-type calmodulin lacking $Ca^{2+}$-binding activity, the correct conformation is present nevertheless, but in our Phe → Ala mutants the correct conformation now requires $Ca^{2+}$ binding. This idea might be testable by combining the Phe → Ala mutations with the mutations that eliminate $Ca^{2+}$ binding.

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