Vertebrate and yeast calmodulin, despite significant sequence divergence, are functionally interchangeable

(calcium-binding protein/*Saccharomyces cerevisiae*/overproduction)

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ABSTRACT Yeast strains relying solely on vertebrate (*Xenopus laevis*) calmodulin, expressed under control of a yeast (*GAL1*) promoter, grew at the same rate as yeast cells containing their endogenous calmodulin. Therefore, the ability to perform essential functions has been conserved between yeast and vertebrate calmodulins, suggesting that calmodulin performs the same (or overlapping) roles in yeast as it does in higher eukaryotes. Successful substitution of vertebrate for yeast calmodulin also suggests that the two proteins can adopt similar conformations *in vivo*, despite the large number of amino acid differences between them (60 out of 148 residues). Strains overproducing either vertebrate or yeast calmodulin about 100-fold and a strain producing a normal level of yeast calmodulin were essentially indistinguishable in many characteristics, including microtubule distribution, rate of secretion, response to mating pheromone, sporulation, and adaptation to nutrient limitation. Calmodulin overproduction did not confer elevated resistance to a phenothiazine drug, trifluoperazine, thought to be a calmodulin-specific inhibitor. These results have important implications for understanding the role of calmodulin in intracellular calcium signaling.

Calmodulin is a small calcium-binding protein that is thought to be responsible for transducing changes in intracellular calcium level into a wide variety of cellular responses (1). The amino acid sequences of calmodulins described from the animal, plant, and protist kingdoms share 85% or greater identity (1, 2). Although its biochemical properties *in vitro* are similar to these other proteins, calmodulin from the yeast *Saccharomyces cerevisiae* shares only 60% identity with the primary structure of other calmodulins (3, 4). Because we have shown previously that deletion of the yeast gene encoding calmodulin (*CMD1*) is lethal (3), we could test whether the calmodulin of a higher organism was capable of supporting yeast growth.

Here we describe the construction of plasmids that permitted the regulated expression of both a vertebrate (frog) calmodulin gene and the authentic yeast (*S. cerevisiae*) gene in yeast cells. The ability of the vertebrate protein to support growth, metabolism, and other cellular processes was then examined. Because the expression system used resulted in substantial overproduction of both the vertebrate and yeast proteins, the physiological consequences of elevating the cellular content of calmodulin by two orders of magnitude could also be assessed.

MATERIALS AND METHODS

Cells, Plasmids, and Growth Media. All strains used in this work were derived from diploid yeast strain TDY21 (*MATa*/*MATa ade2-1^{oc}/ADE2 ADE5/ade5 HIS3/his3-Δ200 his6/*

HIS6 leu2-3,112/leu2-3,112 lys2-333/lys2Δ::HIS3 sst1/SST1 trp1-289/trp1-Δ901 ura3-52/ura3-52 cmd1Δ::TRP1/CMD1 gal/GAL). Standard methods were used for DNA-mediated transformation, sporulation, and tetrad dissection (5). Both rich medium (YP; growth medium containing 1% yeast extract and 2% peptone) and minimal medium lacking uracil or lysine (for the selection and maintenance of plasmid-containing cells), containing either 2% (wt/vol) galactose or 2% (wt/vol) glucose, were as described (5).

Plasmid pTD39 expressing frog calmodulin under *GAL1* promoter control (6, 7) was constructed by using a *Xenopus laevis* calmodulin cDNA from plasmid C71 (8). The cDNA was excised by digestion of C71 with *Pst* I, and the resulting fragment was inserted into the *Pst* I site in the polylinker of the replicative form of M13mp9 (9). The resulting plasmid was linearized by digestion with *Hind*III, and the vector sequences on the 5' side of the cDNA were resected by digestion with BAL-31 nuclease, using standard methods (10). After incubation with *Escherichia coli* DNA polymerase I, the blunt ends so generated were ligated to synthetic *Bam*HI linkers. The DNA was diluted, religated, and used to transform *E. coli*. From several of the transformants obtained, single-stranded DNA was prepared and subjected to nucleotide sequence analysis (11). In this way, a clone was identified in which the initiator ATG of the cDNA was positioned 16 base pairs downstream of the *Bam*HI adapter. Replicative-form DNA was prepared from this clone and cleaved by digestion with *Bam*HI and *Sal* I, and the resulting fragment was purified by agarose gel electrophoresis. The purified fragment was ligated into a yeast *GAL* expression vector, pBM258 (same as pBM150, described in ref. 7), that had been digested with both *Bam*HI and *Sal* I. In the resulting construct (pTD39), which was confirmed by nucleotide sequence analysis, the ATG of the *Xenopus* calmodulin coding sequence lies 82 base pairs downstream from the start site for transcription in the *GAL1* promoter (7). To construct plasmid pEL1, the yeast *CMD1* gene was excised from plasmid pTD30 (3) and inserted into pBM258 in a similar fashion. In pEL1, the ATG of the yeast calmodulin coding sequence lies 104 base pairs downstream from the *GAL1* transcription start site. Plasmid pTD28 consists of the *CMD1* gene inserted into a 2-μm circle-based vector that carries the *LYS2* gene (12) as the selectable marker.

Construction of Control Strains. Yeast *cmd1Δ::TRP1* haploids carrying pTD39, which also contained either the *lys2-333* or the *lys2Δ::HIS3* mutation, were transformed with pTD28. *Lys*⁺ transformants that had lost the plasmid carrying the *Xenopus* calmodulin gene (pTD39) were identified by replica-plating individual colonies onto minimal medium lacking uracil. To generate cells producing yeast calmodulin at its normal level, *Lys*[−] derivatives then were selected by

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Abbreviation: TFP, trifluoperazine.

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using the α -amino adipate method (12). A small fraction of these Lys⁻ isolates represent cells capable of losing pTD28 because gene conversion of the chromosomal *cmd1* Δ ::*TRP1* locus by the *CMD1* DNA on the plasmid had occurred. Hence, such cells become Trp⁻. These derivatives were identified by replica-plating the Lys⁻ isolates onto medium lacking tryptophan. To generate cells overproducing yeast calmodulin from the *GAL1* promoter, the cells carrying pTD28 were retransformed to Ura⁺ with pEL1. Lys⁻ derivatives from which the pTD28 plasmid had been evicted were then selected by using the α -amino adipate method. The genotypes of the final strains were confirmed by restriction enzyme cleavage and Southern filter hybridization analysis of total DNA by using standard methods (5, 10).

Calmodulin Purification and Assay. Strains were grown at 30°C in YP containing 2% galactose to a density of 200 units on a Klett–Summerson photoelectric colorimeter (equipped with a no. 66 red filter). Even though these growth conditions were not selective for the nutritional marker carried by the *GAL1* vector, there was still selection for maintenance of the plasmids expressing either vertebrate or yeast calmodulin because the cells carried a *cmd1* null mutation. After harvesting and washing, lysates were prepared and clarified by low-speed centrifugation. Equal amounts of protein from each extract were loaded onto separate columns of phenyl-Sepharose (Pharmacia) by the methods we have described (3). The columns were washed and eluted as before (3). Samples of the eluate fractions were boiled briefly in SDS gel buffer and subjected to electrophoresis in a slab of 14% polyacrylamide containing SDS. The gel was stained with Coomassie brilliant blue. Samples of the purified proteins were also assayed for their ability to stimulate the activity of a calmodulin-dependent enzyme, bovine brain cyclic nucleotide phosphodiesterase (1), by using 3',5'-[³H]cAMP as the substrate under the conditions described by Wallace *et al.* (13), with the following modifications. Reactions were quenched by boiling, and the product (5'-[³H]AMP) was resolved from unreacted substrate by chromatography on thin layers of polyethyleneimine cellulose on plastic sheets using 0.2 M LiCl as the solvent and quantitated by liquid scintillation spectroscopy of appropriate pieces of the chromatogram. Protein concentration was determined by the microbiuret method (14).

Growth Inhibition by 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10H-phenothiazine (Trifluoperazine; TFP). Exponentially growing cultures of the strains to be tested were diluted to a density of 20 Klett units in YP containing 2% galactose. A portion of each suspension (0.2 ml) was mixed with 1.25 ml of the same medium and 1.25 ml of molten 1.5% agar (45°C) and immediately poured onto the surface of a Petri dish containing solid YP with 2% galactose. After the top agar had solidified, sterile filter disks (6-mm diameter; Difco) containing either 4 or 5 μ l of a 20 mM stock solution of TFP (Boehringer Mannheim) in dimethyl sulfoxide were placed on the surface. The plates were incubated at 30°C until the lawns were clearly visible (2–3 days), and the diameters of the zones of growth inhibition ("halo") were measured. Dimethyl sulfoxide alone did not detectably inhibit growth.

RESULTS AND DISCUSSION

A cDNA encoding the calmodulin of the amphibian *X. laevis* was placed under the control of the promoter of the yeast *GAL1* gene. This promoter is induced when cells are grown in medium containing galactose and repressed when cells are grown in medium containing glucose (6, 7). A plasmid (pTD39) carrying this construction, as well as the *URA3* gene (15) as a selectable marker and a centromere to confer efficient mitotic segregation in yeast (16), was introduced by

DNA-mediated transformation using standard methods (5) into a diploid yeast strain (TDY21) homozygous for the *ura3-52* mutation and heterozygous for a null mutation in the *CMD1* gene (*cmd1* Δ ::*TRP1*/*CMD1*) (3). Ura⁺ transformants of the diploid were sporulated. Twenty of the resulting asci were dissected onto YP medium containing 2% galactose, and the phenotype of the haploid spores was analyzed. Nineteen of the viable spores were Trp⁻ (because they possessed the intact copy of the *CMD1* locus); 5 of these were Ura⁺ and 14 were Ura⁻, as expected for the independent segregation of the centromere plasmid. However, five of the viable spores were Trp⁺ and Ura⁺. Thus, these spores contained the mutant *cmd1* allele, but were alive, presumably because the vertebrate calmodulin provided by the *Xenopus* gene on the plasmid substituted for yeast calmodulin in performing those functions essential for viability. Two observations supported this conclusion. First, no Trp⁺ Ura⁻ spores were obtained. Second, none of the Trp⁺ Ura⁺ spores could grow on medium containing glucose, a condition that prevents expression of the *GAL1* promoter.

Because of their survival, the cells relying on the vertebrate calmodulin could be examined further to determine if they were normal or detectably defective in other physiological processes. It is generally the case, however, that the *GAL1* promoter causes high-level expression when fully induced (6, 7, 17). It was important, therefore, to distinguish between those effects on cell behavior that might be attributed to some difference or deficiency in the function of the vertebrate calmodulin and those effects that might actually be due to overproduction of calmodulin *per se*. For this purpose, pairs of isogenic control strains were derived directly from the cells relying on vertebrate calmodulin by the procedure described in *Materials and Methods*. One control strain expressed yeast calmodulin from its normal chromosomal locus; the other overproduced yeast calmodulin from the *GAL1* expression vector.

To determine the relative levels of calmodulin produced, lysates were prepared from the strain relying on vertebrate calmodulin and from its isogenic control strains. Calmodulin was isolated from each extract, starting with an equal amount of total cellular protein, by using the rapid single-step procedure described previously (3). As revealed by SDS/polyacrylamide gel electrophoresis, *Xenopus* calmodulin produced in yeast comigrated with calmodulin purified from a vertebrate source (bovine brain) (Fig. 1). As shown previously (3), yeast calmodulin migrated significantly faster than its vertebrate counterpart, another manifestation of its amino acid sequence divergence. Densitometric scanning of the stained gel showed that the strain carrying the *GAL1*–*CMD1* plasmid overproduced yeast calmodulin at a level about 80-fold higher than that found in the strain expressing *CMD1* from its normal chromosomal locus. Assuming that staining of the vertebrate protein is not dramatically different from the yeast protein, it appeared that the strain carrying the *GAL1*–*Xenopus* calmodulin cDNA plasmid produced vertebrate calmodulin at a level about 170-fold above that found in a normal yeast cell. The yeast calmodulin purified from the overproducer had a specific activity similar (within a factor of 2) to that of yeast calmodulin purified from the normal cells, as measured by its ability to activate a calmodulin-dependent indicator enzyme (bovine brain cyclic nucleotide phosphodiesterase) (data not shown). By the same assay, the vertebrate protein isolated from yeast had a specific activity about 50-fold higher than yeast calmodulin, in agreement with previous reports comparing the efficacy of purified vertebrate and fungal calmodulins in activating vertebrate indicator enzymes *in vitro* (18, 19).

Remarkably, cells relying on vertebrate calmodulin grew at a rate that was virtually the same (within experimental error) as that displayed by wild-type yeast cells grown in galactose

FIG. 1. Analysis of calmodulin overproduction by SDS/polyacrylamide gel electrophoresis. Calmodulin was isolated from the indicated strains by phenyl-Sepharose chromatography and analyzed by electrophoresis in a polyacrylamide gel in the presence of SDS. Equal volumes of the eluate fraction were loaded in lanes B and C; a 4-fold greater volume of the eluate fraction was loaded in lane D than was applied in lanes B or C. Lane A, molecular weight standards: bovine brain calmodulin (bbCaM) and egg white lysozyme (Lyz); lane B, *Xenopus* calmodulin (xCaM) isolated from strain TDY21-6A carrying pTD39; lane C, yeast calmodulin (yCaM) isolated from strain TDY21-6A carrying pEL1; lane D, calmodulin isolated from strain TDY21-6AC expressing yeast calmodulin from the *CMD1* locus on chromosome II.

medium (Fig. 2). The strain overproducing yeast calmodulin displayed only a modest reduction (15%) in growth rate compared to normal cells. Furthermore, after a shift to rich medium containing glucose (instead of galactose), the cells relying on vertebrate calmodulin continued to grow expo-

FIG. 2. Growth rate of yeast cells relying on heterologous and homologous calmodulin. The growth rate of normal yeast cells (wild type), yeast cells relying on a vertebrate calmodulin (*Xenopus*), and yeast cells overexpressing the *CMD1* gene (overproducer) in YP containing 2% galactose at 30°C was measured by following the increase in turbidity (in Klett units). The strains used were the same as those described in the legend to Fig. 1. The data shown represent seven independent determinations for the normal cells (average doubling time = 184 ± 7 min; mean $\pm$ range), six independent determinations for the cells relying on *Xenopus* calmodulin (average doubling time = 195 ± 6 min), and five independent determinations for the cells overproducing yeast calmodulin (average doubling time = 211 ± 3 min).

entially until cell number increased 80- to 100-fold (in two independent trials), whereupon growth ceased abruptly. Because the *GAL1* promoter is not operative in glucose medium and the vertebrate protein was present at a level about 170-fold above normal prior to the shift, the increase in cell number achieved suggests that vertebrate calmodulin can support yeast growth even when its level has fallen (by growth dilution) to about 2-fold the level found in a normal yeast cell. Although the doubling time of the strain relying on vertebrate calmodulin appeared to be relatively normal, it was possible that this heterologous calmodulin might cause some other detectable perturbation in cellular metabolism and thereby provide clues as to the critical functions of calmodulin in yeast.

There are numerous reports that calmodulin associates with cytoskeletal structures in animal cells, especially microtubules (20). As visualized by indirect immunofluorescence with anti-yeast tubulin antibodies (21, 22), the pattern and distribution of microtubules in the cells relying on vertebrate calmodulin were indistinguishable from those in cells containing yeast calmodulin at either the normal or the high level (data not shown). Calcium has been implicated in the regulation of secretion in both animal cells (23) and yeast (24). Isogenic *MAT α* strains relying on vertebrate calmodulin or on yeast calmodulin at its normal level or at an overexpressed level released a similar amount (11 ± 3 ng per 10^6 exponential phase cells) of α -factor mating pheromone, a well-characterized product of the yeast secretory pathway (25), as judged by an agar diffusion bioassay (26) using known amounts of synthetic α factor as the standards.

Ca^{2+} influx has been implicated in the response of yeast cells to mating pheromone (27, 28). Isogenic *MAT α* cells relying on *Xenopus* calmodulin or on yeast calmodulin at its normal level or at an overexpressed level displayed the same ($\pm 4\%$) sensitivity to α -factor-induced growth arrest, as judged by the agar diffusion bioassay (data not shown). Furthermore, a patch mating test (5) showed that all three strains were capable of mating with virtually the same efficiency, and a *MAT α /MAT α* diploid homozygous for the *cmd1 Δ ::TRP1/cmd1 Δ ::TRP1* mutation, but relying on vertebrate calmodulin expressed from pTD39, sporulated with essentially the same efficiency as an otherwise isogenic *CMD1/CMD1* diploid (data not shown).

Perturbation of cellular regulatory pathways can result in an inability of yeast cells to adapt appropriately to nutrient limitation. For example, an overactive cAMP signaling system results in a rapid loss of viability in stationary phase and a markedly increased sensitivity to killing by heat shock (29, 30). Isogenic strains relying on vertebrate calmodulin or on yeast calmodulin expressed at its normal level or at an overexpressed level survived starvation for a nitrogen source equally well and displayed essentially identical sensitivities to heat shock (data not shown).

The only difference detected between cells relying on vertebrate calmodulin and those depending on yeast calmodulin was in cell size. The distribution of cell volumes in asynchronous cultures was measured in a fluorescence-activated cell sorter for cells in exponential phase and stationary phase, with or without fixation for 1 hr in 3.7% formaldehyde. Regardless of the state of the culture examined, the mean size of the cells relying on vertebrate calmodulin was 10–20% larger than the mean size of the control cells.

We also tested whether overproduction of calmodulin conferred resistance to the phenothiazine drug TFP, which is known to bind to calmodulin *in vitro* and prevent the activation of calmodulin-dependent enzymes (31). It has been demonstrated for a variety of enzymes that overproduction *in vivo* can confer cellular resistance to a specific inhibitor (32). Perhaps the best known example of this dosage effect is

Table 1. Inhibition of yeast growth by TFP

Genotype	Halo diameter with TFP, mm	
	80 nmol	100 nmol
<i>CMD1</i>	10.4 ± 0.5	11.5 ± 0.5
<i>cmd1Δ</i> [p <i>Xenopus</i> -CaM]	10.3 ± 0.8	11.2 ± 1.0
<i>cmd1Δ</i> [p <i>CMD1</i>]	9.3 ± 0.6	10.1 ± 0.9

The strains used were the same as those indicated in the legend to Fig. 1. The values given represent the mean ± SD of determinations performed in triplicate.

methotrexate resistance conferred by overproduction of dihydrofolate reductase (33). Yeast cell growth is inhibited by TFP (34–36). If the only target for TFP *in vivo* is calmodulin, then overproduction of the protein should confer elevated resistance. In marked contrast to this expectation, strains overproducing either yeast or vertebrate calmodulin were no more resistant, within experimental error, than a wild-type yeast strain (Table 1). This observation indicates that, in yeast, some other target is more sensitive to TFP than calmodulin; therefore, studies analyzing the effects of TFP on yeast cells (36, 37) may not provide any physiologically relevant insight into calmodulin function *in vivo*. In agreement with this view, mutations in three different genes (35, 38) can confer phenothiazine resistance in *S. cerevisiae*, but none of these loci correspond to the *CMD1* gene, which is closely linked to *LYS2* on chromosome II (unpublished results). Perhaps similar caution should be used in attributing the effects of phenothiazine drugs in animal cells to inhibition of calmodulin; indeed, other important cellular regulatory proteins have been shown to be affected by phenothiazines *in vitro*, including protein kinase C (39) and a calpactin (40).

The fact that vertebrate calmodulin could substitute for yeast calmodulin in yeast cells suggests that the functions of calmodulin have been highly conserved during the long epoch (approximately a billion years) since yeast and present-day vertebrates must have diverged from their common progenitor (41). Other vertebrate proteins have been shown to substitute for their counterparts in yeast, including *ras* protooncogene products (42), the human homologue of the *CDC28/CDC2* protein kinase (43), mouse ribosomal protein L27' (44), and hamster hydroxymethylglutaryl-CoA reductase (45). In several of these other cases, however, the cells relying on the vertebrate protein display some phenotypic differences from normal yeast cells, which suggests that the vertebrate protein is less than optimal. In contrast, yeast cells relying on vertebrate calmodulin grew just as well as a wild-type yeast strain and showed no other detectable aberration in cellular physiology (except for a slightly larger cell size). Because the biological function of proteins requires a specific three-dimensional structure, vertebrate and yeast calmodulins must be able to adopt very similar conformations *in vivo*, despite the fact that they differ in 60 out of 148 positions (3).

Overproduction of structural proteins often has dramatic effects on cells because proper assembly of complex structures requires correct stoichiometric ratios of the components. In yeast, for example, overproduction of an individual histone species causes a dramatic decrease in the fidelity of chromosome transmission during mitosis (46); similarly, only a 2-fold elevation in the level of β -tubulin is toxic (47), and overproduction of actin is lethal (L. Cheever and D. Drubin, personal communication). In contrast, overproduction of either vertebrate or yeast calmodulin had little or no apparent effect on yeast cell physiology. Although calmodulin has been reported to be associated with the cytoskeleton (20, 48) and the mitotic spindle (49) in animal cells, our results in yeast suggest that these associations, if truly present, are most likely regulatory, rather than structural, interactions.

The fact that overproduction of calmodulin had so little effect is perhaps surprising. Rasmussen and Means (50, 51) have observed that a 2- to 4-fold elevation of the calmodulin level in mouse C127 cells reduces the cell cycle time by shortening the G_1 period. In contrast, we found that an 80-fold elevation of the yeast calmodulin level slightly increased the yeast cell division time. Consistent with this conclusion, we also found that yeast cells relying on vertebrate calmodulin, produced at a level 170-fold greater than normal, had a slightly larger average size, which is also diagnostic for a condition that lengthens the cell division time of yeast cells (52).

Assuming that yeast cells are able to maintain a constant level of free cytosolic calcium, equilibrium considerations predict that an 80-fold increase in yeast calmodulin should produce a correspondingly large increase in the level of the liganded forms of calmodulin, including the Ca_3^{2+} - and Ca_4^{2+} -bound states thought to be responsible for activation of calmodulin-dependent enzymes (1). Thus, even in the absence of any signal that raises the resting level of Ca^{2+} , which in yeast is about 200–400 nM (J. Kao, M. Hasson, J.T., and R. Tsien, unpublished results), calmodulin overproduction might have resulted in the constitutive activation of calmodulin-dependent pathways. Inappropriate stimulation of other regulatory systems in yeast (for example, constitutive activation of the cAMP pathway) is certainly deleterious (29, 30). Strikingly, however, calmodulin overproduction did not seem to have any markedly adverse effects.

One explanation for the lack of effect of yeast calmodulin overproduction might be that a substantial fraction of the protein was nonfunctional. This possibility is unlikely because the overproduced calmodulin bound and eluted from the affinity column in a Ca^{2+} -dependent manner and was able to activate a calmodulin-dependent enzyme with a specific activity that was the same (within experimental error) as that of the protein isolated from normal cells.

Because it is not known what minimum level of "free" cytosolic Ca^{2+} is sufficient to support the growth of yeast or any other eukaryotic cell, an alternative explanation for the lack of effect of calmodulin overproduction might be that calmodulin participates in a regulatory feedback loop that maintains the intracellular Ca^{2+} concentration at an appropriate level. For example, yeast cells may possess calmodulin-dependent Ca^{2+} pumps in the plasma (53) and/or vacuolar (54) membranes that are responsible for extruding Ca^{2+} from the cytosol. The Ca^{2+} -ATPase of the red blood cell plasma membrane is just such a calmodulin-dependent Ca^{2+} pump (55). A third alternative to explain our observations is that changes in the level of the Ca^{2+} /calmodulin complex, rather than its absolute concentration, may be responsible for modulating cellular functions. This situation would require that a mechanism(s) exist that allows for adaptation or desensitization over time of the targets of calmodulin action. Perhaps modulation of the degree of covalent modification of targets mediated by a calmodulin-dependent protein kinase (56) and a calmodulin-dependent phosphoprotein phosphatase (57), operating at different relative rates, might provide such an adaptation mechanism.

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