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Calmodulins with deletions in the central helix functionally replace the native protein in yeast cells

(evolution/calcium-binding proteins/*Saccharomyces cerevisiae*)

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ABSTRACT **Deletion of Glu-84, Glu-83 and Glu-84, or Ser-Glu-Glu-Glu (residues 81–84) from the central helix of mammalian calmodulin is known to result in a 5–7 times decrease in its apparent *in vitro* affinities for three calmodulin-dependent enzymes. However, based on *in vitro* experiments, it is difficult to estimate how these deletions might affect *in vivo* cellular function. The yeast *Saccharomyces cerevisiae*, which requires calmodulin for growth, provides an excellent system to evaluate these deletion proteins *in vivo*. Based on its ability to restore normal growth characteristics to yeast cells, mammalian calmodulin is functionally identical to the yeast protein; herein we evaluate the effect of deleting residues 84, 83 and 84, or 81–84 from the central helix. Sequences encoding the deletion proteins and an unaltered control sequence were introduced by means of a yeast shuttle vector and were expressed under control of the yeast calmodulin promoter. The deletion and control calmodulins are produced at levels similar to that observed for the yeast protein, and they completely restore normal growth characteristics. This result suggests that the regions deleted from the central helix are not critical for activation of any yeast calmodulin target normally required for cell growth or division. It is likely that there are twisting and shortening motions associated with the deletions from the central helix that alter significantly the spatial relationship between the two lobes of calmodulin. The abilities of the deletion calmodulins to restore completely normal growth characteristics to yeast cells suggest that the lobes of all the deletion proteins can still be appropriately positioned in calmodulin–target complexes. This is consistent with the hypothesis that the central helix of calmodulin is analogous to a flexible tether rather than to a rigid connector between the two lobes of the molecule.**

In eukaryotes, calmodulin is a key mediator of responses to stimulus-induced changes in the intracellular concentration of calcium ions. The three-dimensional structure of $(Ca^{2+})_4$-calmodulin has been refined at 2.2-Å resolution (1). The structure consists of two globular lobes connected by a 42-Å central helix in which the central third is completely exposed to solvent. Each lobe contains a pair of EF-hand calcium-binding domains. There is no contact between the two lobes in the crystal structure. Two-dimensional nuclear magnetic resonance analyses of the protein indicate that there is also little contact between the lobes in solution (2). However, small-angle x-ray scattering measurements (3) and biochemical investigations (4, 5) are consistent with flexibility of the central helix in solution. In one study it was shown that a pair of cysteines substituted at positions more than 25 Å apart in the crystal structure can cross-link the two lobes of calmodulin in solution by means of a disulfide bond (4).

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Molecular modeling (6), biochemical (4, 5, 7), nuclear magnetic resonance (8), and small-angle x-ray scattering investigations (9, 10) suggest that bending within the central helix is functionally important at least in part because it allows the two lobes of calmodulin to interact simultaneously with some of the short peptides and target domains that it binds with high affinity. Proteolytic cleavage of the central helix separates the two lobes and destroys high-affinity interactions between calmodulin and several targets (11, 12). This effect of cleavage can be reversed by forming a chemical cross-link between the two lobes (4). Although it does not resemble the central helix, the cross-link appears to reproduce its tethering function. These observations led to the proposal that the central helix functions as a flexible tether between the two lobes (4–6).

There are currently 25 known calmodulin amino acid sequences (13); they reflect a remarkable degree of conservation over very significant evolutionary distances. Four representative calmodulin sequences are presented in Fig. 1. Conservation of primary structure in the central helix of calmodulin suggests that performing as a flexible tether is only part of its functional repertoire. This idea is supported by a variety of studies showing that alterations in the length and composition of the central helix have *in vitro* functional consequences (5, 18–20).

We have investigated (5) the *in vitro* functional properties of three engineered calmodulins in which Glu-84, Glu-83 and Glu-84, and Ser-Glu-Glu-Glu (residues 81–84) are deleted from central helix. As seen in Fig. 1, this region is well conserved even in yeast, which is the most divergent calmodulin so far discovered. The *in vitro* functional properties of the three deletion proteins are very similar. All activate myosin light chain kinase, calcineurin, and plant NAD kinase activities to the same relative levels, which are within 20% of control values (5). All the deletions cause a 5 to 7 times reduction in apparent affinities for the three evaluated enzymes and for a peptide representing the calmodulin-binding domain of myosin light chain kinase (5). The lack of any significant differences among the three deletion proteins suggests a great degree of tolerance to changes in the length of the central helix of calmodulin and to accompanying changes in the spatial relationship between the two lobes. Whereas this observation is consistent with a high degree of flexibility within the central helix, it appears inconsistent with the exact conservation in the number of residues seen in the central helices of all known calmodulins (Fig. 1).

One possible explanation is that, although these deletion proteins may be functional with a few targets *in vitro*, they would not perform adequately *in vivo*. This could be because the relatively small functional changes we have observed in *in vitro* assay systems would not be tolerated *in vivo* or because of a significant decrease in the ability of calmodulin to activate one or more enzymes not evaluated by us *in vitro*.

Abbreviation: hCaM, yeast–mammalian hybrid calmodulin.

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Human    1  ADQLTEEQ-IAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARK
Anemone  1  ADQLTEEQ-IAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGdGTIDFPEFLTMMARK
Wheat    1  ADQLTdEQnIAEFKEAFSLFDKDGDGcITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLnLMARK
Yeast    1  ssnLTEEQ-IAEFKEAFaLFDKDnnGsIsssELaTVMRSLGlsPsEAEvnDlmNEiDvDGNhqIeFsEFLaLMsRq

Human    76  MKDTDSEEEIREAFRVFDKDGnGyISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVqMMTAK
Anemone  76  MKDTDSEEEIREAFRVFDKDGDGFISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVKMMTSK
Wheat    77  MKDTDSEEELkEAFRVFDKDqDGFISAAELRHVMTNLGEKLTDEEVDEMIREADvDGDGQINYEEFVKvMmAK
Yeast    76  lKsnDSEqELlEAFkVFDKnGDGlISAAELkHVlTsiGEKLTDaEVDdMlRE-vsDGsGeINiqqFaall-SK
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FIG. 1. Comparison of four representative calmodulin amino acid sequences; human (CAMHS; ref. 14), sea anemone (CAMMS; ref. 15), wheat (CAMT; ref. 16), and *S. cerevisiae* (CAMSC; ref. 17). Identity between two sequences is indicated by a vertical line. Residues that are unique to a given calmodulin are in lowercase letters. Asterisks indicate residues that coordinate the Ca^{2+} ion. Lines are drawn over the α-helical regions; the double line indicates the central helix. The region in the central helix from which deletions were made, residues 81–84, is indicated by vertical arrows.

More indirect consequences of the deletions could also come into play. For example, the stability or availability of calmodulin might be affected by alterations in the central helix, thereby reducing its intracellular concentration below the level necessary for full function.

By using relatively simple techniques, yeast strains can be constructed in which the native calmodulin gene has been deleted and the only source of calmodulin is that expressed from an introduced template (17, 21). Yeast cells lacking a calmodulin gene are not viable (17); however, calmodulin expressed from a mammalian template restores normal growth characteristics (21, 22). In this study we evaluate whether calmodulins in which residues 84, 83 and 84, or 81–84 have been deleted from the central helix also can restore growth to calmodulin-deficient yeast cells.

METHODS

The DNA templates encoding control and deletion calmodulins were put under control of the promoter for the yeast calmodulin gene (*CMD1*) to ensure that they would be produced at a level near that at which calmodulin is normally

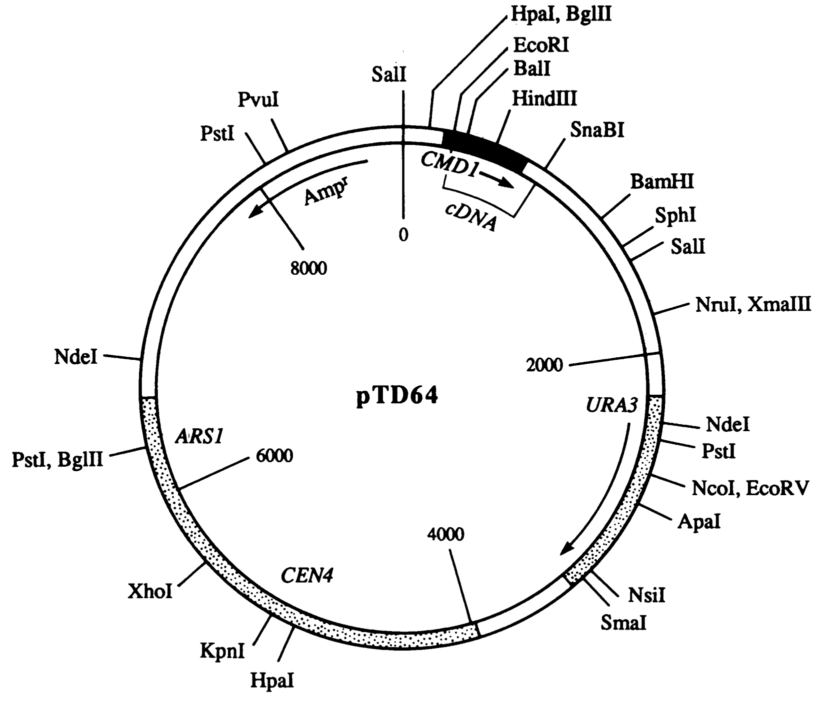

FIG. 2. Construction of the plasmids encoding control and deletion calmodulins. Plasmid pTD64 contains the intact *CMD1* gene. In plasmids pTD65, pTD66, pTD67, and pTD68, the majority of the *CMD1* gene has been replaced with a cDNA encoding mammalian calmodulin or mammalian calmodulins with residues 84, 83 and 84, or 81–84 deleted from the central helix, respectively. Sites are shown for restriction enzymes that have a 6-base recognition sequence and cut the plasmid three or fewer times.

produced in yeast cells. The parent plasmid is the yeast shuttle vector pTD64, which is a derivative of plasmid YCp50 and contains *CMD1* in addition to the *URA3*, *CEN4*, and *ARS1* genes, which are required for maintenance of the plasmid in yeast. In plasmid pTD64 the fragment from the *Eco*RI site 350 base pairs upstream of the initiation codon of the *CMD1* gene to the *Stu* I site 505 base pairs downstream from the stop codon of the *CMD1* gene is inserted in place of the *Eco*RI–*Bam*HI fragment of YCp50. The *Eco*RI site 350 base pairs upstream was replaced with a *Sal* I site by performing a partial digest with *Eco*RI, filling-in with the Klenow fragment of DNA polymerase I, ligating *Sal* I linkers to the blunt ends, and recircularizing the plasmid with T4 DNA ligase. Thus, in the plasmid pTD64, there is only one *Eco*RI site at position +35 relative to the initiation codon in the *CMD1* gene (Fig. 2). The *Eco*RI–*Sna*BI fragment containing the majority of the *CMD1* gene was then replaced with the *Eco*RI–*Sph* I fragments of the pKK233-2 plasmids (5) containing DNA templates encoding either unaltered mammalian calmodulin or one of the three deletion proteins. All plasmids were checked by sequencing the entire region encoding calmodulin (23). In these constructions the regions encoding the first 10 amino acid residues of calmodulin are derived from the yeast *CMD1* gene; therefore, the first 3 amino acids in the final protein products are Ser-Ser-Asn as is found in yeast calmodulin rather than Ala-Asp-Gln as is found in the mammalian protein (see Fig. 1). Sequences encoding four calmodulins were introduced in yeast cells. These proteins are termed hCaM, the control yeast–mammalian hybrid calmodulin; hCaMΔ84, hCaM with Glu-84 deleted from the central helix; hCaMΔ83-84, Glu-83 and Glu-84 deleted; and hCaMΔ81-84, Ser-Glu-Glu-Glu (residues 81–84) deleted. Steady-state levels of calmodulin in yeast cell lysates were measured as described (21). Transformation of yeast strains was performed as described elsewhere (21).

RESULTS AND DISCUSSION

Plasmids carrying the DNA templates encoding hCaM, hCaMΔ84, hCaMΔ83-84, or hCaMΔ81-84 were transformed into diploid yeast strain TDY49, which has the following genotype: *MATa/MATα*, *ade2-loc/ade2-loc*, can1-100/can1-100, *cmd1Δ::TRP1/CMD1*, *his3-11,15/his3-11,15*, *leu2-3,112/leu2-3,112*, *lys2Δ::HIS3/lys2Δ::HIS3*, *trpl-1/trpl-1*, *ura3-1/ura3-1*. Ura$^+$ transformants were selected and sporulated and the tetrads dissected (24). As suggested by previous results (21, 22), diploids transformed with the plasmid encoding hCaM give rise to viable haploid cells in which the endogenous *CMD1* gene is deleted (cells were trp$^+$) and the only source of calmodulin is the cDNA encoding hCaM. Four out of 6 spores that received the plasmid (ura$^+$) were also trp$^+$ and, therefore, received the *cmd1Δ::TRP1* allele. This proportion of trp$^+$ spores is as predicted assuming independent segregation of *cmd1Δ* and the plasmid carrying the *URA3* gene plus the templates encoding calmodulin. No spores that carried the *cmd1Δ::TRP1* allele (trp$^+$) and lacked the plasmid (ura$^-$) survived. Diploids transformed with a construct encoding hCaMΔ84, hCaMΔ83-84, or hCaMΔ81-84 also give rise to viable haploid cells: 11 out of 21 ura$^+$ spores, 3 out of 9 ura$^+$ spores, and 2 out of 4 ura$^+$ spores, respectively. Haploid yeast cells relying on hCaM, hCaMΔ84, or hCaMΔ83-84 have the same growth rates in rich liquid medium as do cells with the normal amount of yeast calmodulin (Fig. 3). Haploid yeast cells relying on hCaMΔ81-84 show about a 10% decrease in growth rate. On solid minimal medium (24), all the strains form the same size colonies at 30°C.

Although the sequences encoding hCaM, hCaMΔ84, hCaMΔ83-84, and hCaMΔ81-84 are under the control of the promoter for the yeast gene, their steady-state levels could, as a result of post-transcriptional processes, be different from the steady-state level of yeast calmodulin. So we measured the steady-state levels of calmodulin produced in strains relying on the control and deletion calmodulins and found in each case that the amount of calmodulin is within 2-fold of that in yeast cells carrying the *CMD1* gene at its normal locus. Levels of expression of hCaM, hCaMΔ84, hCaMΔ83-84, and hCaMΔ81-84 were, respectively, 0.7-, 0.6-, 1.2-, and 1.3-fold the level of yeast calmodulin.

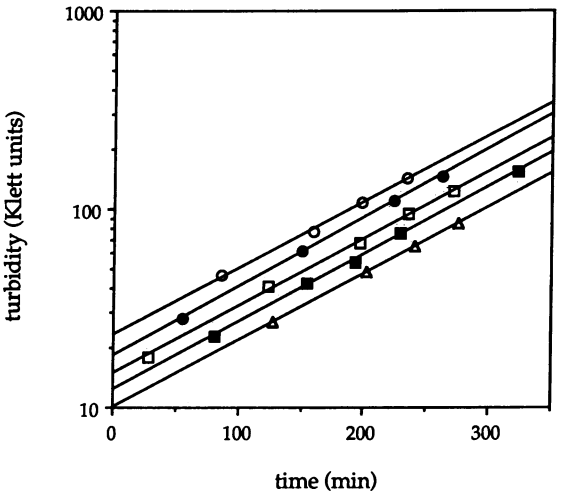

FIG. 3. Growth of yeast strains relying on mutant calmodulins. ○, A yeast strain in which the yeast *CMD1* gene is present at its normal chromosomal locus (strain TDY49-3B); ●, a yeast strain deleted for the *CMD1* gene and carrying plasmid pTD65, which encodes hCaM (strain TDY49-3C); □, a yeast strain deleted for the *CMD1* gene and carrying plasmid pTD66, which encodes hCaMΔ84 (strain TDY49-17D); ■, a yeast strain deleted for the *CMD1* gene and carrying plasmid pTD67, which encodes hCaMΔ83-84 (strain TDY49-7C); △, a yeast strain deleted for the *CMD1* gene and carrying plasmid pTD68, which encodes hCaMΔ81-84 (strain TDY49-12B). Yeast strains were grown at 30°C in liquid YP medium (24) containing 2% glucose. The cell density was measured in a Klett–Summerson meter equipped with a 66 filter. These curves are representative of the results of growth curves performed in triplicate. The average (± SD) generation time for strains with *CMD1* at its normal chromosomal locus was 84 ± 5 min. For strains deleted for the *CMD1* gene and carrying plasmid pTD65, pTD66, pTD67, or pTD68, the average generation times were 89 ± 4 min, 92 ± 4 min, 89 ± 3 min, or 94 ± 1 min, respectively.

We have demonstrated that calmodulins in which Glu-84, Glu-83 and Glu-84, or Ser-Glu-Glu-Glu (residues 81–84) have been deleted from the central helix can functionally replace the native protein in *Saccharomyces cerevisiae* when produced in comparable amounts. Although there are both structural and functional differences between yeast and mammalian calmodulins (25, 26), the numbers of residues in the central helices of the yeast and mammalian proteins are exactly the same as the region altered in the deletion proteins; residues 81–84, Ser-Glu-Glu-Glu, is almost identical to yeast calmodulin residues 81–84, Ser-Glu-Gln-Glu (17). However, under the conditions used in this study the indicated deletions from the central helix do not affect in a functionally significant way the binding or activation of any calmodulin target necessary for the growth and division of yeast cells. It has been shown *in vitro* that identical deletions decrease the apparent affinities of mammalian calmodulin for plant NAD kinase, myosin light chain kinase, and calcineurin by factors of 5–7 (5). If binding or activation of any yeast target necessary for cell growth and division is similarly affected, this does not result in an obvious phenotype. However, in evolutionary terms we must conclude that under some conditions yeast strains dependent on the deletion proteins are at a competitive disadvantage compared with a

strain dependent on the unaltered protein. A very slight disadvantage, which might be difficult to observe, could be definitive on an evolutionary time scale.

Modeling of small-angle x-ray scattering data indicate that in the deletion proteins the spatial relationship between the two lobes of calmodulin is altered due to twisting and shortening of the central helix (27). As reviewed elsewhere (6), there is good evidence that formation of several calmodulin–target complexes depends on interactions involving both lobes of calmodulin. If this also applies to yeast calmodulin–target complexes involved in cell growth and division, then the apparent lack of any effect of the deletions supports the hypothesis that the central helix of calmodulin is not a rigid connection between the two lobes but is more analogous to a flexible tether (4–6).

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