J. Biochem. 2012;152(1):27–35 doi:10.1093/jb/mvs048

JB THE JOURNAL OF BIOCHEMISTRY

Specific conformation and Ca^{2+}-binding mode of yeast calmodulin: insight into evolutionary development

Received January 18, 2012; accepted February 24, 2012; published online May 4, 2012

Kenichi Nakashima*†, Hiroaki Ishida†, Akiko Nakatomi and Michio Yazawa

Graduate School of Science, Hokkaido University, N10-W8, Sapporo, Hokkaido 060-0810, Japan

*Kenichi Nakashima, Obihiro University Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan. Tel: +81-155-49-5430, Fax: +81-155-49-5229, email: knakashima@obihiro.ac.jp

†Present addresses: Kenichi Nakashima, Obihiro University Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, 080-8555, Japan; Hiroaki Ishida, Biochemistry Research Group, Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4, Canada

The vertebrate calmodulin is configured with two structurally independent globular lobes in N- and C-terminus, and a flexible central linker. Distinctly, two lobes of calmodulin from *Saccharomyces cerevisiae* (yCaM) interact and influence the Ca^{2+}-binding profile of each other. We explored this further using the mutant proteins with eliminated Ca^{2+}-binding ability in one of the lobes and found that the Ca^{2+}-bound N-lobe associates with the Ca^{2+}-free C-lobe to gain the Ca^{2+} affinity of a wild-type level. Next, analysing series of C-terminal residue truncation mutant, we found that the truncation of C-terminal three residues induce the hyper Ca^{2+} affinity. These residues are also important for the general structural behaviour of calmodulin, such as Ca^{2+}-induced slow mobility shift in polyacrylamide gel electrophoresis and for the ability to activate Cmk1p (yeast calmodulin kinase). These suggest: (i) when Ca^{2+} occupies only N-lobe, two lobes interact and form the stable intermediate leading to a proper level of Ca^{2+} affinity; (ii) the C-terminal three residues are required to prohibit abnormal stabilization of the intermediate promoting abnormally high Ca^{2+} affinity and for recognition of target enzymes. A model for Ca^{2+} and target bindings of yCaM is proposed. Evolutional aspect concerning the biological significance of this model was discussed.

Keywords: calcium-binding protein/calmodulin/conformation/evolution/yeast.

Abbreviations: CaM, calmodulin; FITC, fluorescein isothiocyanate; LC20, regulatory light chain of smooth muscle myosin isolated from chicken gizzard; MOPS, 3-morpholinopropanesulfonic acid; PMSF, penylmethane sulfonylfluoride; vCaM, vertebrate calmodulin; WGD, whole genome duplication; yCaM, calmodulin from *Saccharomyces cerevisiae*.

Calmodulin is a Ca^{2+}-binding protein, which senses the change in intracellular Ca^{2+} concentration, and activates multiple target enzymes in various eukaryotic cell types (*1*). Amino acid sequence of calmodulin is conserved over 90% of identity in every other organism except for several ascomycota fungi [identity to the vertebrate calmodulin (vCaM): *Saccharomyces cerevisiae* (61%), *Kluyveromyces lactis* (61%), *Ashbya gossypii* (61%), *Candida albicans* (72%), *Neurospora crassa* (85%) or *Schizosaccharomyces pombe* (75%)]. The vCaM contains four Helices E-F of troponin C (EF) hand motifs (site I, II, III and IV) to give ligands for Ca^{2+} (*2*). Each two EF hand motifs of the N- or the C-terminus make a pair and each pair forms globular domain called the N-lobe or the C-lobe (*3*). The central region between the N-lobe and the C-lobe serves as a flexible linker (*4*), and dynamically bends when calmodulin binds to a target (*5–7*). A significant difference between the vCaM and yeast calmodulin from *S. cerevisiae* (yCaM) is seen in the Ca^{2+}-binding site IV, alteration in two highly conserved amino acid, Asp129 (a loss) and Glu140 (Gln instead), therefore Ca^{2+} capacity of yCaM is three versus four of vCaM (*8*, *9*).

Notably, the N-lobe and the C-lobe of yCaM cooperatively binds Ca^{2+}, in contrast to that two lobes of vCaM independently bind Ca^{2+} (*10*). Indeed, the conformation of the C-lobe observed for the peak of C2 proton of His107 (in the middle of C-lobe) in ^{1}H-NMR, changes when Ca^{2+} associates with the N-lobe (*10*, *11*). Therefore, it has been thought that Ca^{2+}-bound N-lobe interacts with the C-lobe and improves Ca^{2+} binding of the C-lobe. The experiment using half-molecular fragments of yCaM indicated that the inter-lobe interaction also improves Ca^{2+} affinity of the N-lobe (*10*). Calmodulin from other type of yeast *S. pombe* also indicates that the intermediate conformation with His107 and two lobes interplay Ca^{2+}-binding profile (*12*).

The tertiary structures of Ca^{2+}-free form of full-size protein or Ca^{2+}-bound form of N-terminal half molecule of yCaM are not significantly diverse from vCaM (*11*, *13*). The structure of Ca^{2+}-bound form of the full-size yCaM has not been determined because of the flexible structure of the site IV. Instead, by removing the site IV, Ca^{2+}-bound form of yCaM (truncation form) has been determined and a globular conformation by the direct hydrophobic interaction between N-lobe and site III has been shown (*14*). This suggests that Ca^{2+}-binding profile of one lobe is indeed affected by the Ca^{2+} binding of the other lobe. In general, when Ca^{2+} is associated with calmodulin, each lobe changes the conformation to expose hydrophobic

amino acid residues, which in turn causes association of amphiphilic peptide of target enzymes, removal of the auto-inhibitory domain and further rearrangement of the active site. Target enzyme activation activity of yCaM is extremely weak when the phosphodiesterase and myosin light chain kinase from vertebrate tissues are examined (*7*, *15*, *16*). It is possible that the mode to activate target enzymes with yCaM is somewhat different from vCaM. However, there is only a little study has been performed for the activation mechanism of yCaM except for that indicating spreading phenylalanine residues act for target binding (*17*), although there are more than 10 CaM-binding proteins in *S. cerevisiae* (*18*).

Interestingly, vCaM and calmodulin from *S. pombe* can substitute for yCaM in proliferation of *S. cerevisiae* cells (*19*, *12*). Moreover, yCaM mutant without Ca^{2+} binding ability still supports the proliferation (*20*). However, yCaM cannot support proliferation of *S. pombe* (*12*), suggesting that the essential target enzymes of Ca^{2+}-bound CaM kept in *S. pombe* were lost in *S. cerevisiae* in the evolution.

In this study, we show the mode of Ca^{2+} binding for non-canonical calmodulin from yeast and propose a mechanism by which it activates target enzyme. Evolutional aspect concerning the biological significance was discussed for these differences.

Materials and Methods

Sample preparation

yCaM (wild type), yCaMEEQ (formerly E104Q; yCaM with disrupted Ca^{2+}-binding site III in the C-lobe) and yCaMΔ15″ (formerly YCMΔ; with truncated 15 amino acids at the C-terminus) were described earlier (*10*, *11*). yCaMQQE (E31Q E67Q), in which the essential glutamate of sites I and II was replaced by glutamine to eliminate Ca^{2+} binding ability, was obtained from *Escherichia coli* BL21(DE3) transformed with pETyCaMQQE generated by the PCR-based site-directed mutagenesis (*21*). A series of C-terminal truncation mutants was obtained from BL21(DE3) transformed with pETyCaMΔ1, pETyCaMΔ2, pETyCaMΔ3, pETyCaMΔ6 or pETyCaMΔ9, each with a generated termination codon at the corresponding site of pETyCaM using conventional site-directed mutagenesis method.

The recombinant yeast calmodulin derivatives were purified using the trichloroacetic acid precipitation method with some modifications (*22*). yCaMEEQ and yCaMQQE were purified under the same conditions used for preparation of yCaM using 0.4 M ammonium sulphate-containing buffer to bind to the phenyl Sepharose column (*23*). All C-terminal truncation mutants were purified using 0.25 M ammonium sulphate-containing buffer in the phenyl Sepharose chromatography step. Fractions containing yCaM derivatives were subjected to the anion exchange column chromatography (*10*). The proteins were eluted with a linear gradient of NaCl concentration of 0.05–0.35 M, and yCaM derivatives were eluted around 0.25 M NaCl. The purified proteins were desalted with gel filtration and then lyophilized. They were stored at −20°C until use.

Ca^{2+}-binding analysis

Ca^{2+}-binding measurement was performed using the flow-dialysis method at 25°C in the buffer containing 0.1 M NaCl, 20 mM 3-morpholinopropanesulfonic acid (MOPS) (pH 7.0) as described earlier (*16*, *24*). Data were collected from three separate experiments for each sample and all data points were plotted to calculate dissociation constant by curve fitting to the Adair's equation until $R^2 > 0.999$. The equations used in this analysis were described earlier (*10*, *16*).

Cmk1p purification and assay

Following purification steps were performed at 4°C. To isolate the calmodulin-dependent protein kinase (Cmk1p) from *S. cerevisiae*, 100 g of live yeast pellets (Nitten Co) were suspended in 150 ml of buffer A [0.1 M Tris–Cl (pH 7.5), 1 mM EGTA, 14 mM 2-mercaptoethanol and 1 mM penylmethane sulfonylfluoride (PMSF)]. The suspension was treated with French press (SLM-Aminco) at a pressure of 12,000 psi to disrupt cells and the resulting homogenate was centrifuged at 150,000*g* for 1 h. The supernatant was collected and mixed with 100 ml of wet DE32 (Whatman) resin, which was equilibrated and suspended with buffer B [20 mM Tris–Cl (pH 7.5), 1 mM EGTA, 14 mM 2-mercaptoethanol and 1 mM PMSF] containing 0.05 M NaCl. After 30 min of incubation, the mixture was poured into the column (5 cm diameter) and the settled resin was washed with 1 l of buffer B containing 0.05 M NaCl. The adsorbed proteins were eluted with buffer B containing 0.1 M NaCl and the fractions containing high kinase activity were collected. Ammonium sulphate was added to the collected solution to 50% of saturation. After 30 min of incubation, it was centrifuged at 15,000*g* for 20 min. The supernatant was adjusted to 70% saturation of ammonium sulphate. After 30 min of incubation, it was again centrifuged at 15,000*g* for 20 min. The resulting precipitate was suspended with buffer B containing 0.5 M NaCl and dialyzed against the same buffer. The dialyzed protein solution was adjusted to 1 mM $CaCl_2$ and applied to a yCaM-conjugated Sepharose 4B column equilibrated with buffer C [20 mM Tris–Cl (pH 7.5), 0.5 M NaCl, 0.1 mM $CaCl_2$, 14 mM 2-mercaptoethanol and 1 mM PMSF]. The column was washed with five bed volumes of buffer C and eluted with buffer C containing 1 mM EGTA instead of 0.1 mM $CaCl_2$. The solution was dialyzed against buffer B containing 50% glycerol and 0.1 M NaCl and stored at -20°C after the dialysis.

To assay the kinase activity, the regulatory light chain of smooth muscle myosin (LC20) isolated from chicken gizzard was used as substrate. Reaction mixture [10 nM Cmk1p, 0.5 mg/ml substrate, 1 mM $CaCl_2$, 20 mM MOPS (pH 7.0), 0.1 M NaCl, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol and various concentrations of calmodulin derivatives] was added to 2 mM ATP to start the reaction at 25°C. Reaction was terminated with 8 M urea, and the reaction products were analysed using 8 M urea-15% polyacrylamide gel electrophoresis (*25*).

Gel overlay of FITC-labelled yCaM derivatives

To label with fluorescein isothiocyanate (FITC), 1 mg of lyophilized yCaM, yCaMEEQ, yCaMQQE or yCaMΔ15″ was dissolved in 0.25 ml of buffer containing 0.1 M borate (pH 8.4) and 75 mM NaCl. To the protein solution, 0.5 mg FITC powder was added and the solution was incubated at 20°C for 2 h. The reaction was terminated by adding 25 µl of 1 M ethanolamine. Remaining FITC was removed by Sephadex G25 (40 ml bed volume) gel filtration with buffer containing 20 mM imidazole and 0.1 M NaCl. The peak fractions were collected and the protein concentration was measured with Bio-Rad Protein Assay Kit (Bio-Rad). Purified Cmk1p (0.1 µg) was subjected to 8% SDS–PAGE and the gel with migrated protein was washed with water for 1 h. The gel was soaked in Tris-buffered saline containing 0.5 µM FITC-CaM and 0.1 mM $CaCl_2$ or 0.1 mM EGTA. Gel was incubated at 20°C overnight and then washed with the consistent buffer except FITC-CaM was eliminated. FITC-CaM bound to the protein was visualized using the UV transilluminator.

^{1}H-NMR

The ^{1}H NMR spectra were obtained at a frequency of 600 MHz using a JEOL JNM-A600 spectrometer to monitor the signals of the C2 proton of the His residue. The details of experimental condition are described in Nakashima *et al.* (*10*).

Results

Ca^{2+} binding and inter-lobe interaction

The Ca^{2+} affinity of the equimolar mixture of half-molecular fragments of yCaM is lower than that of the full-length protein, suggesting that the interaction between the N-lobe and the C-lobe is required

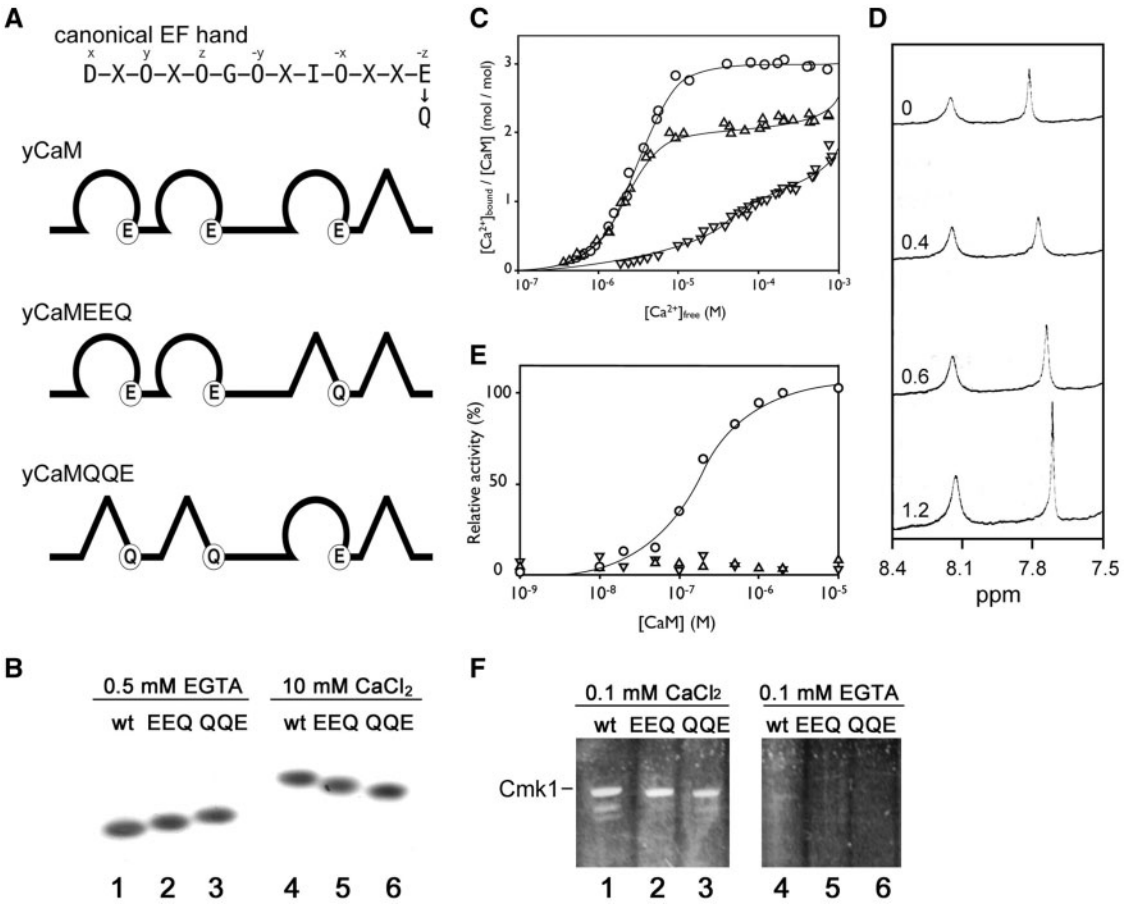

Fig. 1 Analysis of yCaM mutant which binds Ca^{2+} to one of lobes. (A) Canonical EF-hand structure is shown in top (D, aspartate; X, residues not specified; O, residues containing oxygen; G, glycine; I, isoleucine; E, glutamate; Q, glutamine). Amino acids indicated (x, y, z, -x, -y, -z) are Ca^{2+} ligands. Figures indicate primary structures of yCaM and derivatives, yCaMEEQ and yCaMQQE. Loops are perfect EF-hand motifs and triangle roofs are imperfect motifs either the fourth site of yCaM or the site, in which glutamate at -z position is substituted with glutamine. (B) Native PAGE of yCaM wild type, yCaMEEQ or yCaMQQE in the presence of 0.5 mM EGTA (lanes 1–3) or 10 mM $CaCl_2$ (lanes 4–6). (C) Ca^{2+} binding of yCaM (○), yCaMEEQ (△)and yCaMQQE (▽). Assay was performed using flow-dialysis method as described in Materials and methods. Molar ratio of $[Ca^{2+}]_{bound}$ to [CaM] is plotted against $[Ca^{2+}]_{free}$. Lines are drawn by hand. (D) ^1H NMR spectral change of yCaMQQE by Ca^{2+} addition. The signals from the C2 proton of His61 in the N-domain and His107 in the C-domain are appeared around 8.15 and 7.8 ppm, respectively. The measurement was performed in a medium of 50 mM KCl and 50 mM MOPS/KOD at pH 7.5. The molar ratios of $[Ca^{2+}]_{added}$ to [yCaMQQE] are shown. (E) Activation of Cmk1p with yCaM (○), yCaMEEQ (△)and yCaMQQE (▽). Assay was performed as described in Materials and methods. Myosin light chain (LC20) was used as the substrate. Data were normalized to the activity value when 1 μM of yCaM was added. (F) Overlay of FITC-labelled yCaM derivatives to the gel in which Cmk1p was separated. The purified Cmk1p was applied on a SDS PAGE. The gel was incubated with FITC-yCaM (gels 1 and 4), FITC-yCaMEEQ (gels 2 and 5) or FITC-yCaMQQE (gels 3 and 6) in the presence of 0.1 mM $CaCl_2$ (gels 1–3) or 0.1 mM EGTA (gels 4–6) as indicated.

for proper Ca^{2+} binding for yCaM (*10*). It is not clear whether Ca^{2+}-bound form or free form of a half-molecular lobe is effective for the Ca^{2+} binding of the other half molecular lobe. To examine this, we prepared yCaM mutants with Ca^{2+}-binding capacity limited to the N-lobe or to the C-lobe by substituting the critical glutamate residue at -z position in the EF-hand motif to the neutral glutamine residue [yCaMEEQ (E104Q) or yCaMQQE (E31Q E67Q)] (Fig. 1A).

First, we analysed the Ca^{2+}-dependent mobility change of these mutants on native PAGE (Fig. 1B). On one hand, in the absence of Ca^{2+}, they run slower with increasing number of E to Q substitution. On the other hand, they run faster with increasing the number of E to Q substitution in the presence of 0.5 mM Ca^{2+}. All of them showed slower mobility by addition of Ca^{2+} as canonical calmodulin does (*26*).

Maximum number of Ca^{2+} bound to yCaMEEQ and yCaMQQE was expected to be two and one equivalent to protein, respectively (Fig. 1C). However, Ca^{2+} binding of yCaMQQE was not saturated at one Ca^{2+} per protein. Because the E to Q mutation of the corresponding site is known to reduce Ca^{2+} affinity by several hundred folds (*27*), probably Ca^{2+} binding of the mutated N-lobe in yCaMQQE is observed when more than 0.1 mM Ca^{2+} were present. To see if Ca^{2+} binds first to the C-lobe in this mutant, we examined the conformation change of C-lobe by the Ca^{2+} titration. The NMR peak of C2 proton of His107 in the C-lobe shifts to lower field when the N-lobe is occupied with Ca^{2+} and the peak shifts to higher field when the C-lobe is occupied with Ca^{2+} (*10*). In the case of yCaMQQE, high-field shift was observed when Ca^{2+} equivalent to a protein was added to yCaMQQE, indicating that non-disrupted site III of the C-lobe has higher Ca^{2+}

affinity than disrupted sites I and II in the N-lobe (Fig. 1D). As described earlier, Ca^{2+} affinity of the N-lobe fragment (Y12) is 4–5-fold lower than that of wild-type yCaM (*10*). In contrast, yCaMEEQ showed same level of Ca^{2+} affinity as that of wild-type yCaM (Fig. 1C), indicating that the appropriate Ca^{2+} affinity of the N-lobe is retained if the molecular property other than the impaired Ca^{2+} binding capacity in the C-lobe remained intact. On the other hand, the Ca^{2+} affinity of yCaMQQE was ~5-fold lower than the anticipated Ca^{2+}-binding curve of the C-lobe, which is obtained as the difference of Ca^{2+}-binding curve of wild type and that of yCaMEEQ. Therefore, when the N-lobe is not occupied with Ca^{2+}, the Ca^{2+} affinity of the C-lobe does not attain the wild-type level.

Although site IV of vCaM plays an important role for activation of target enzymes (*28*), site IV of yCaM is faulty in its EF-hand motif, and disable to bind Ca^{2+}. Therefore, it has been thought yCaM has a distinct mechanism to activate target enzymes. To examine this, we used one of the yeast specific calmodulin kinase Cmk1p purified from *S. cerevisiae* cells with LC20 as a substrate, Cmk1p showed ~10 folds lower activity value than that of myosin light chain kinase from chicken gizzard. Although, K_{act} value of yCaM is ~100 nM, which is very high when compared with the K_{act} value of vCaM (5.1 nM for chicken gizzard myosin light chain kinase, 2.4 nM for rabbit skeletal muscle myosin-light-chain kinase, 0.2 nM for scallop calcineurin and 1.4 nM for porcine brain phosphodiesterase) (Fig. 1E) (*16*), it is applicable to evaluate yCaM. We found that yCaMEEQ and yCaMQQE failed to activate Cmk1p (Fig. 1E). In spite of their lack of ability in Cmk1p activation, these mutants were able to bind Cmk1p in a Ca^{2+}-dependent manner, which was observed by gel-overlay assay using FITC-labelled yCaM or mutant protein (Fig. 1F). Therefore, if one of the lobes binds Ca^{2+}, yCaM is able to bind Cmk1p, but it is not sufficient to activate the enzyme.

yCaM requires several amino acid residues at C-terminus for proper range of Ca^{2+} affinity and activation of Cmk1p

We have shown that yCaM C-terminal truncation mutant (deletion of 15 residues in the C-terminus with two alternative residues, YCMΔ132-148, renamed yCaMΔ15″ in this study) indicates higher Ca^{2+} affinity than wild-type yCaM (Fig. 2C) (*10*, *23*), suggesting that the C-terminus of yCaM plays a role in inter-lobe interaction to modify Ca^{2+}-binding ability (*29*). To know how many C-terminal amino acid residues are required, we constructed a series of C-terminal truncation mutants (1, 2, 3, 6 and 9 residues were deleted from the C-terminus) and compared with wild-type yCaM and yCaMΔ15″ (Fig. 2A). We observed that the more the deletion, the faster the mutant migrates on urea PAGE under denatured condition (Fig. 2B). It is expected that same pattern of migration is observed with these mutants on native PAGE, if there is no conformational difference. However, under Ca^{2+}-free (5 mM EGTA) condition, distinctly yCaMΔ1 migrates slower than wild type, yCaMΔ2 migrates even slower than yCaMΔ1 and yCaMΔ3 migrates slower than yCaMΔ2 (Fig. 2B). Mutant with more than four residues truncated induces faster migration by each diminishing step as seen in urea PAGE. These results suggest that conformational disruption continues with deleting amino acid one by one, until three from the C-terminus of yCaM. It has been shown that hydrophobic core of several phenylalanine residues inside of the each lobe is exposed to the solvent, when Ca^{2+} bind to calmodulin. Assuming that the slow migration shift is linked to the exposure of the hydrophobic core of the protein, the C-terminal three residues of yCaM are possibly required for concealing the hydrophobic core in the absence of Ca^{2+}.

Ca^{2+} binding and activation of Cmk1p by the C-terminal truncation mutants were then examined. Interestingly, deleting amino acid one by one from the C-terminus, increased the Ca^{2+} affinity and deleting three amino acids was almost sufficient to cause the affinity level similar to yCaMΔ15″ (Fig. 2C). The macroscopic dissociation constants were estimated using these data processed with the Adair's equation (*10*). K_2 value is getting smaller causing higher Ca^{2+} affinity by the truncation, although K_1 and K_3 are not affected much (Fig. 2D), suggesting that cooperativity of the first two Ca^{2+} bindings is increased and the third Ca^{2+} binding occurs apparently independent of the truncation. In addition, truncations of two, three and four amino acids drastically reduced the target activation activity (higher K_{act} and lower V_{max}; Fig. 2E). Thus, the three C-terminal amino acid residues (Leu-Ser-Lys-COOH) act to control Ca^{2+} binding traits of whole molecule, and, especially, Leu-Ser within the region also plays an important role for full activation of Cmk1p. Alteration of the yCaM traits is likely linked to the migration shift on the native PAGE, the apparent conformational disruption. When yCaMΔ15″ was subjected to the gel-overlay experiment, the mutant indicated a much weaker signal than wild type did (Fig. 4F). This suggests that C-terminal amino acid residues of yCaM play a role for proper target association and following activation.

Discussion

Ca^{2+}-binding mode of non-canonical calmodulin from yeast

The mode of Ca^{2+} binding of yCaM has been revealed to be more complicated than that of vCaM, because inter-lobe interaction modifies overall Ca^{2+} affinity (*10*, *11*, *14*). It is still unclear about mechanical details of Ca^{2+} binding of yCaM. In this study, we found several additional clues for this issue. First, it is not necessary for the C-lobe to bind Ca^{2+} to induce the wild-type level of high Ca^{2+} affinity of the N-lobe. Second, it is necessary for the N-lobe to bind Ca^{2+} to induce high Ca^{2+} affinity of the C-lobe. Third, C-terminal three amino acid residues are involved in the regulation of overall Ca^{2+} affinity and target

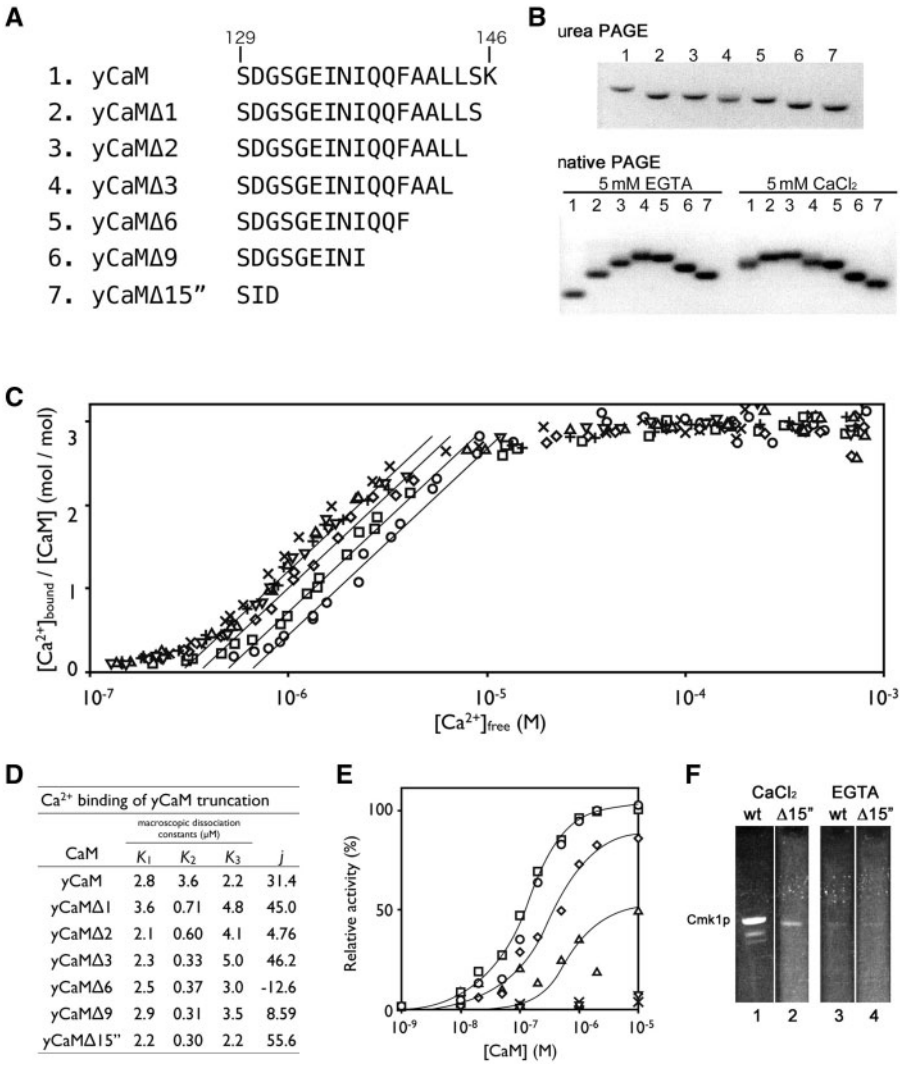

Fig. 2 Analysis of C-terminal truncation mutant of yCaM. (A) 1: Amino acid sequence of the C-terminal 18 residues (129–146) of yeast calmodulin. 2–6 are yCaM truncated mutants (yCaMΔ*n*, *n* is the number of amino acid residues deleted from the C terminus). 7 is identical to YCMΔ132-148 (Matsuura *et al.*, 1991). (B) Results of electrophoresis of mutants. Top, urea PAGE and bottom, native PAGE. Experiments were performed as shown in Materials and methods. Sample numbers indicate those given in A. Samples subjected to native PAGE were adjusted to 5 mM EGTA or 5 mM $CaCl_2$ as indicated. (C) Ca^{2+} binding to the mutant shown in (A). Data were processed as shown in Fig. 1. yCaM (○), yCaMΔ1 (□), yCaMΔ2 (◇), yCaMΔ3 (△), yCaMΔ6 (▽), yCaMΔ9 (+) and yCaMΔ15″ (×). The lines indicate exponential Ca^{2+} binding of yCaM (right), yCaMΔ1 (second from right), yCaMΔ2 (second from left) and others (left). (D) The macroscopic dissociation constants (K_1, K_2 and K_3) which give best-fit curve to the Adair's equation for the corresponding Ca^{2+} binding data of Fig. 2C. Data were dealt as described previously (*10*). (E) Activation of Cmk1p with yCaM C-terminal truncation mutants. yCaM (○), yCaMΔ1 (□), yCaMΔ2 (◇), yCaMΔ3 (△), yCaMΔ6 (▽), yCaMΔ9 (+) and yCaMΔ15″ (×). Lines were drawn by hand. (F) Overlay of FITC-labelled yCaMΔ15″ to the gel in which Cmk1p was separated. Experiment was performed as in Fig. 1F, except the gel was incubated with FITC-yCaM (gels 1 and 3) or FITC-yCaMΔ15″ (gels 2 and 4) in the presence of 0.1 mM $CaCl_2$ (gels 1 and 2) or 0.1 mM EGTA (gels 3 and 4) as indicated.

activation. We are considering the underlying mechanism for these findings as follows:

(1) How does the C-lobe induce high Ca^{2+} affinity of the N-lobe without Ca^{2+}? The tertiary structure of Ca^{2+}-free yCaM is indicated that two lobes do not interact with each other (*11*). On the other hand, in the presence of Ca^{2+}, two lobes of yCaM are associated and form overall globular conformation, if the C-terminal EF-hand is truncated (*14*). The vCaM also forms a globular conformation when it binds to a target peptide and induces high Ca^{2+} affinity, because the affinity of peptide and CaM is very high ($K_d = 10^{-10}$ to 10^{-9} M) and the chemical equilibrium largely shifts towards the Ca^{2+}-bound state (*30*). Both globular conformations are formed based on the interaction between the hydrophobic core of each lobe and therefore the chemical equilibrium shift may be occurred in yCaM to induce high Ca^{2+} affinity even in the absence of target peptide. The conformational transition to the stable globular shape likely occurs when only N-lobe is occupied with Ca^{2+}, because even yCaMEEQ shows wild-type level of Ca^{2+} affinity and also the C-lobe conformation change was observed by the shift of His107 peak in ^{1}H-NMR Ca^{2+} titration analysis (*7*, *10*, *11*).

(2) How does the N-lobe induce the high-Ca^{2+} affinity of the C-lobe, only when it is occupied with Ca^{2+}? N-lobe is occupied first by Ca^{2+} and C-lobe is the low affinity site in the case of yCaM (*10*). It was shown that the intermediate conformation of yCaM is appeared when N-lobe is occupied with Ca^{2+} (*11*), and here we have shown that the intermediate conformation is required for wild-type level of high Ca^{2+} affinity for the C-lobe. In the case of Ca^{2+} titration of C-lobe, ^{1}H-NMR peak for C2 proton of His107 (C-lobe) shifts to the lower magnetic field with slow exchange manner (*10*). However, the peak of the corresponding residue shows fast exchange manner in yCaMQQE (Fig. 1D). This indicates that Ca^{2+} association manner of C-lobe is somewhat different depending on the state of Ca^{2+} occupation of N-lobe. Probably, the rearrangement of C-lobe induced by the Ca^{2+} binding to the N-lobe provides easier access for Ca^{2+} to the binding pocket.

(3) How does the C-terminal truncation induce abnormally high Ca^{2+} affinity? Our results indicated that both Ca^{2+} addition and the truncation of three C-terminal amino acids might induce similar conformational change observed as the slow-migration shift in the native PAGE (Figs 1B and 2B). From structural aspect, it has been shown that Ca^{2+} binding induces an exposure of hydrophobic residues to the molecular surface of calmodulin (*11*, *13*, *14*). Possibly these two phenomena are linked, and if so one by one amino acid truncation from the C-terminus induces similar exposure of hydrophobic residues. When only N-lobe is occupied by Ca^{2+} and the intermediate structure is formed, C-lobe in wild-type yCaM changes the conformation. If those hydrophobic residues are always exposed in C-lobes of truncation mutants, they are no need to change the conformation and thus Ca^{2+} binding likely occurs non-cooperatively with the Ca^{2+} occupation of N-lobe. Nonetheless, C-lobe of the C-terminal truncation mutant is stabilized by the interaction with N-lobe to reach wild-type level or even higher level of Ca^{2+} affinity. This may result in the apparent higher Ca^{2+}-affinity of truncation mutants than that of wild-type yCaM.

The conformational flexibility of site IV is shown as the small numbers of Nuclear Overhauser Effect (NOE) are observed for amino acid residues in the site IV region (*14*). The deletion of the region causes severe defect of the target activation ability of yCaM, probably because two lobes are tightly interacted and target is not able to bind with regular mode. Thus, we propose a role of the C-terminal amino acid residues for the target recognition. We hypothesized that the interacted two lobes of yCaM are dynamically disassembled, and reassembled together with the target enzymes, when they are present, resulting in activation of them. Three C-terminal residues probably play a significant role for this reconstruction steps. This mechanism is not conserved in vCaM, as the C-terminal three-residue truncation does not cause severe disruption of target activation ability of vCaM (*31*, *32*). Still, Met residues near the C-terminus of calmodulin have shown to be required for target recognition (*31*, *32*). Thus, even though the acting mechanism is very different, the C-terminal region of both vCaM and yCaM play important roles for target recognition.

Summary of our proposal is depicted in Fig. 3. First, in wild-type yCaM (Fig. 3A), a Ca^{2+} binds to the N-lobe, which in turn changes conformation to expose hydrophobic core (step I). The hydrophobic core of the N-lobe begins to interact with the site III of the C-lobe (step III), in which hydrophobic residues are exposed after the destruction of site IV (step II). The inter-lobe interaction is established and stabilizes the Ca^{2+}-bound conformation and thus all three Ca^{2+} bindings cooperatively occurred (steps IV and V). To activate target enzymes, C-lobe changes its conformation for recognition of target peptide and creates a complex similar to that of canonical calmodulin with target peptide (step VI). Obviously, C-terminal residue truncation mutants omit the step II to induce inter-lobe interaction (Fig. 3B), and show higher Ca^{2+} affinity than the intact protein (Fig. 2C). This suggests that destruction of site IV in yCaM (step II) is limiting the rate of Ca^{2+} binding reaction. However, to associate with a target enzyme, the globular structure created with sites I, II and III is not suitable and the flexible site IV plays an essential role to create a space between two lobes for the target peptide binding (step VI).

Evolutionary aspect for the feature of yCaM function

The phylogenic tree of ascomycota fungi is well established with their widely available DNA sequences (*33*), and reveals the outline of evolutionary principle, such as the whole genome duplication (WGD) and following massive loss (*34*). The subtype of yCaM, which coordinates three Ca^{2+}, is seen in various yeast species including pre-WGD yeast, such as *K. lactis*, *A. gossypii* and *Zygosaccharomyces rouxii* (Fig. 4B). The calmodulin gene (*CMD1*) in those species is located in the expected origin of two types of *CMD1* location in post-WGD yeasts: one is seen in *S. cerevisiae*, another is seen in *Kluyveromyces polysporus* (Fig. 4A). Other relative species of further distance from *S. cerevisiae*, such as *C. albicans*, *N. crassa*, and *S. pombe* have calmodulin coordinating four Ca^{2+} similar to vCaM. Interestingly, *C. albicans* shows neighbouring genes *ALG1* and *HOS1* near the calmodulin gene as seen in *A. gossypii*, although the order and the orientation were opposite (Fig. 4A). Therefore, even before divergence of four Ca^{2+}-binding CaM and three Ca^{2+}-binding CaM, the genes are aligned in similar order, suggesting that calmodulins in this phylum have a common ancestor, nonetheless for the huge divergence such as reduction of maximum Ca^{2+} capacity.

Interestingly, calmodulin from *S. pombe* shows interaction between the N- and C-lobe and the cooperative Ca^{2+} binding similar to calmodulin from *S. cerevisiae* (*12*). Even with this similarity, calmodulin from *S. cerevisiae* did not restore the lethality of

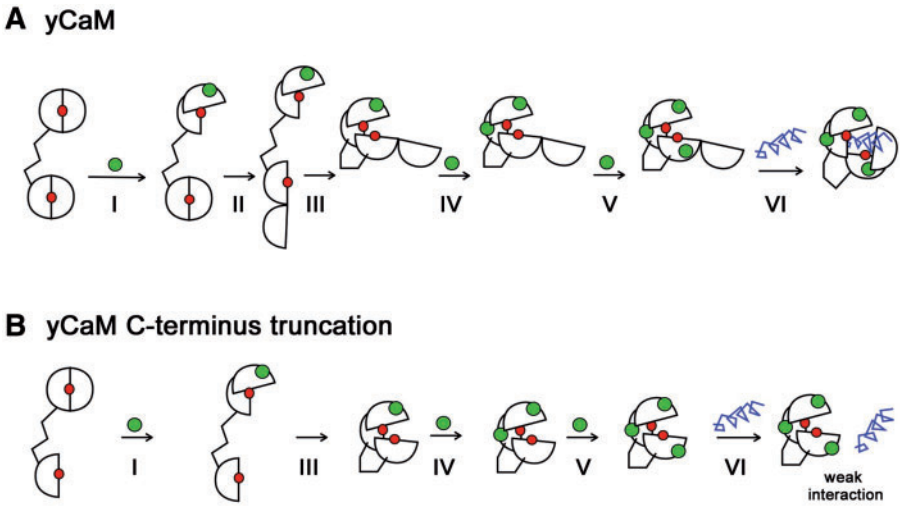

Fig. 3 Working model for Ca^{2+} binding and target activation of yCaM. The model for wild type (A) and the model for a C-terminal truncation mutant (B) are shown. Ca^{2+} is indicated in green and the hydrophobic core of each lobe is indicated in red. Step I, one Ca^{2+} binds to one of the EF-motifs in the N-lobe and cause conformation change to expose the hydrophobic core to solvent. Step II, the C-lobe in closed form equilibrates with that in open form. C-terminal truncation mutants skip this step (B). Step III, hydrophobic interaction between the N-lobe and the C-lobe results in the formation of the globular structure. Steps IV and V, second and third Ca^{2+} binds to the N-lobe and the C-lobe, respectively. Each binding causes conformational alteration in both lobes. Step VI, open form of C-lobe closes and creates the canonical CaM structure and the hydrophobic core of each lobe serves for target recognition and interaction. C-terminal truncation mutants cannot create this structure; hence, the target is hardly recognized with this mutant. The colour version of this figure is available at *JB* online.

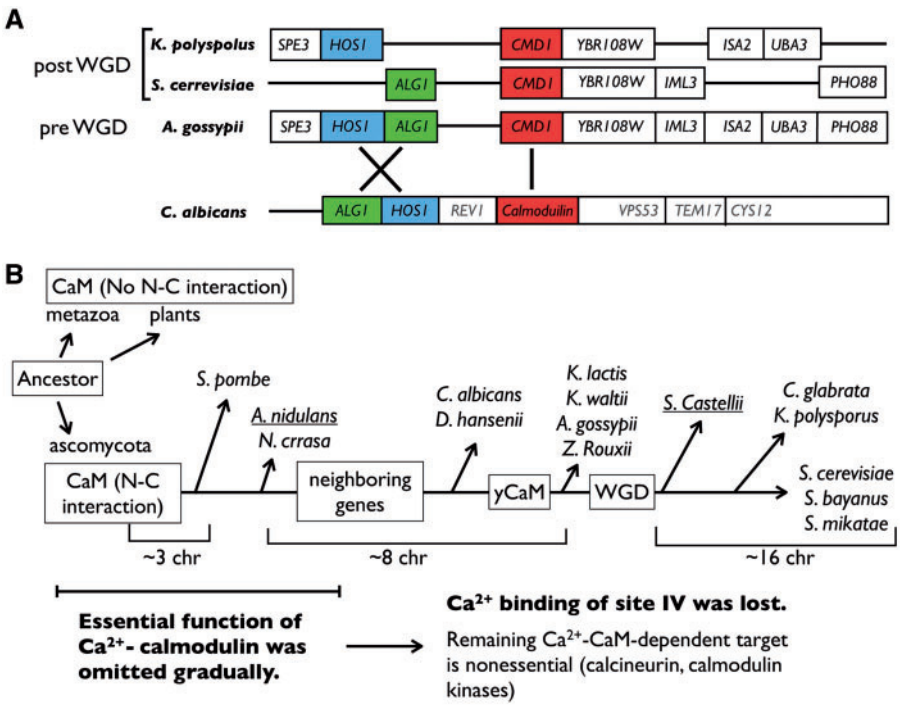

Fig. 4 Evolutionary consideration for yCaM development. (A) Comparison of loci around CaM gene (red) of ascomycota species (*K. polysporus*, *S. cerevisiae*, *A. gossypii* and *C. albicans*). *CMD1* indicates the genes highly homologous to the unique CaM gene (*CMD1*) of *S. cerevisiae*. In *K. polysporus*, *CMD1* is located in the duplicated pair of chromosome at the WGD. In pre-WGD yeast *A. gossypii*, genes of this locus indicate the patchwork of those in *K. polysporus* and those in *S. cerevisiae*. In *C. albicans*, CaM gene generates a four Ca^{2+}-binding CaM and is located more shuffled genes around in addition to two *CMD1* neighbouring genes *ALG1* and *HOS1*, the order and the orientation of which are opposite (green and blue). (B) The development of CaM. When CaM diverged from the common ancestor, metazoan and plant's CaM developed the independent N- and C-lobes. In contrast, CaM in ascomycota including *S. pombe*, far distant species from *S. cerevisiae*, developed N–C interaction. This assumes that the species between *S. pombe* and *S. cerevisiae* contain the CaM with inter-lobe interaction. The approximate total chromosome number is indicated under the timeline. *A. nidulans* and *N. crassa* has similar CaM to vCaM. When *C. albicans* and *Debaryomyces hansenii* developed, CaM gene was relocated to the locus somehow similar to pre-WGD yeast, although the CaM is still similar to that of *S. pombe* or vCaM. Then, three Ca^{2+}-binding CaM (yCaM) was developed. At the same time, four Ca^{2+}-binding CaM was discontinued, probably following to precedent discontinue of the essential Ca^{2+}-CaM target enzymes and thus yCaM is selected in pre-WGD yeasts (*K. lactis*, *Kluyveromyces waltii*, *A. gossypii* and *Z. Rouxii*). Further history of CaM development has shown that *A. nidulans* and *Saccharomyces castellii* have another CaM like gene (indicated by under bar), and *Candida glabrata* and *K. polysporus* has *CMD1* in the locus of pairing duplication at WGD. Cmd1p of *C. glabrata* and *Saccharomyces mikatae* are highly identical to that of *S. cerevisiae*. The colour version of this figure is available at *JB* online.

calmodulin-null cells of *S. pombe*, suggesting different modes of the target activation mechanism (*12*). However, target enzymes and binding proteins in both species are similar at least in our knowledge including, calcineurin, calmodulin kinase, myosin subtypes, IQ motif containing GTPase Activating Protein (IQGAP) and spindle-pole component. The same enzymes are assumed to be under the control of a similar activation mode; hence, it is still possible that specific binding partners of calmodulin may be present in some yeast species. It is required to search comprehensively for the direct binding partner to identify the species-specific Ca^{2+}-CaM target. As Ca^{2+} binding is not essential for yCaM (*19*), it is expected that some essential target enzymes, which require Ca^{2+}-bound form of CaM for activation, are lost before or during development of yCaM. Here is our working model for yCaM development: (Fig. 4B) (i) when a common ancestor separated into metazoa, plants and ascomycota fungi, CaM in fungi group uniquely obtained interactive lobes, (ii) still lots of binding proteins were probably shared between metazoa and yeast near the branch point from the ancestor, so Ca^{2+}-binding ability of CaM was essential for viability in those species, (iii) the essential CaM functions are gradually lost during the evolutionary process in the ascomycota phylum, (iv) Ca^{2+} binding to CaM became no further essential for viability and three Ca^{2+}-CaM was chosen in a evolutionary process at the same time of getting rigid gene order after *C. albicans*, before *A. gossypii*. Targets in *S. pombe* and *C. albicans*, at least, have to be compared with those in *S. cerevisiae* to obtain further insights for this aspect.

In addition, Ca^{2+}-free calmodulin also acts as an essential light chain of metazoan or plant myosin V to create the rigid arm lever. Interestingly, essential light chain of myosin II, Mlc2p, also shows similar phylogenic tree of calmodulin (very large homology gap is seen between *C. albicans* and *A. gossypii*). Hence, they probably experienced synchronized evolutionary process. There are two types of actin organization systems: one is seen in *S. pombe* where microtubules recruit formin at cell pole, and then followed by formation of actin filaments; another is seen in *S. serevisiae* where actin filaments are formed at the cortical marker and microtubules are formed at the tip of bud at mitosis (*35*). Both systems act in *Aspergllus nidulans*, although microtubules system is specifically required for the filamentous growth (*35*). Distinctly, another filamentous yeast *A. gossypii* requires cortical marker for its filamentous growth (*36*). *S. pombe* and *A. nidulans* contains four Ca^{2+} binding CaM, whereas *S. cerevisiae* and *A. gossypii* contains three Ca^{2+} binding CaM. These facts suggest a possible link between functional aspects of actin system in cell morphology and the evolutionary development of calmodulin or myosin essential light chain. Further details are remained to be analysed.

Acknowledgements

We thank Dr Hideto Kuwayama (Obihiro University of Agriculture and Veterinary Medicine) for encouraging us and for incentive discussions. We thank Nitten Co for the kind gift of the cell pellets of baker's yeast.

Funding

This work was supported in part by the Grant-in Aid for Scientific Research (07309013) from the Ministry of Education, Science and Culture in Japan.

Conflict of interest

None declared.

References

1. James, P., Vorherr, T., and Carafoli, E. (1995) Calmodulin-binding domains: just two faced or multi-faceted? *Trends Biochem. Sci.* **20**, 38–42
2. Kretsinger, R.H. and Nockolds, C.E. (1973) Carp muscle calcium-binding protein. II. Structure determination and general description. *J. Biol. Chem.* **248**, 3313–3326
3. Babu, Y.S., Bugg, C.E., and Cook, W.J. (1988) Structure of calmodulin refined at 2.2 Å resolution. *J. Mol. Biol.* **204**, 191–204
4. Zhang, M., Tanaka, T., and Ikura, M. (1995) Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat. Struct. Biol.* **2**, 758–767
5. Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B., and Bax, A. (1992) Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science* **256**, 632–638
6. Meador, W.E., Means, A.R., and Quiocho, F.A. (1992) Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin-peptide complex. *Science* **257**, 1251–1255
7. Meador, W.E., Means, A.R., and Quiocho, F.A. (1993) Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. *Science* **262**, 1718–1721
8. Davis, T.N., Urdea, M.S., Masiarz, F.R., and Thorner, J. (1986) Isolation of the yeast calmodulin gene: calmodulin is an essential protein. *Cell* **47**, 423–431
9. Luan, Y., Matsuura, I., Yazawa, M., Nakamura, T., and Yagi, K. (1987) Yeast calmodulin: structural and functional differences compared with vertebrate calmodulin. *J. Biochem.* **102**, 1531–1537
10. Nakashima, K., Ishida, H., Ohki, S., Hikichi, K., and Yazawa, M. (1999) Calcium binding induces interaction between the N- and C-terminal domains of yeast calmodulin and modulates its overall conformation. *Biochemistry* **38**, 98–104
11. Ishida, H., Nakashima, K., Kumaki, Y., Nakata, M., Hikichi, K., and Yazawa, M. (2002) The solution structure of apocalmodulin from *Saccharomyces cerevisiae* implies a mechanism for its unique Ca^{2+} binding property. *Biochemistry* **41**, 15536–15542
12. Moser, M.J., Lee, S.Y., Klevit, R.E., and Davis, T.N. (1995) Ca^{2+} binding to calmodulin and its role in *Schizosaccharomyces pombe* as revealed by mutagenesis and NMR spectroscopy. *J. Biol. Chem.* **270**, 20643–20652
13. Ishida, H., Takahashi, K., Nakashima, K., Kumaki, Y., Nakata, M., Hikichi, K., and Yazawa, M. (2000) Solution structures of the N-terminal domain of yeast calmodulin: Ca^{2+}-dependent conformational change and its functional implication. *Biochemistry* **39**, 13660–13668

14. Ogura, K., Kumeta, H., Takahashi, H., Kobashigawa1, Y., Yoshida, R., Itoh, H., Yazawa, M., and Inagaki, F. (2012) Solution structures of yeast *Saccharomyces cerevisiae* calmodulin in calcium- and target peptide-bound states reveal similarities and differences to vertebrate calmodulin. *Genes Cells* **17**, 159–172
15. Ohya, Y., Uno, I., Ishikawa, T., and Anraku, Y. (1987) Purification and biochemical properties of calmodulin from *Saccharomyces* cerevisiae. *Eur. J. Biochem.* **168**, 13–19
16. Nakashima, K., Maekawa, H., and Yazawa, M. (1996) Chimeras of yeast and chicken calmodulin demonstrate differences in activation mechanisms of target enzymes. *Biochemistry* **35**, 5602–5610
17. Okano, H., Cyert, M.S., and Ohya, Y. (1998) Importance of phenylalanine residues of yeast calmodulin for target binding and activation. *J. Biol. Chem.* **273**, 26375–26382
18. Cyert, M.S. (2001) Genetic analysis of calmodulin and its targets in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **35**, 647–672
19. Davis, T.N. and Thorner, J. (1989) Vertebrate and yeast calmodulin, despite significant sequence divergence, are functionally interchangeable. *Proc. Natl. Acad. Sci. USA.* **86**, 7909–7913
20. Geiser, J.R., van Tuinen, D., Brockerhoff, S.E., Neff, M.M., and Davis, T.N. (1991) Can calmodulin function without binding calcium? *Cell* **65**, 949–959
21. Ito, W., Ishiguro, H., and Kurosawa, Y. (1991) A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. *Gene* **102**, 67–70
22. Yazawa, M., Sakuma, M., and Yagi, K. (1980) Calmodulins from muscles of marine invertebrates, scallop and sea anemone. *J. Biochem.* **87**, 1313–1320
23. Matsuura, I., Ishihara, K., Nakai, Y., Yazawa, M., Toda, H., and Yagi, K. (1991) A site-directed mutagenesis study of yeast calmodulin. *J. Biochem.* **109**, 190–197
24. Yazawa, M., Vorherr, T., James, P., Carafoli, E., and Yagi, K. (1992) Binding of calcium by calmodulin: influence of the calmodulin binding domain of the plasma membrane calcium pump. *Biochemistry* **31**, 3171–3176
25. Kendrick-Jones, J., Szentkiraliyi, E.M., and Szent-Gyorgyi, A.G. (1976) Regulatory light chains in myosins. *J. Mol. Biol.* **104**, 747–755
26. Grand, R.J., Perry, S.V., and Weeks, A.R. (1979) Troponin C-like proteins (calmodulins) from mammalian smooth muscle and other tissues. *Biochem. J.* **177**, 521–529
27. Maune, J.F., Klee, C.B., and Beckingham, K. (1992) Ca^{2+} binding and conformational change in two series of point mutations to the individual Ca^{2+}-binding sites of calmodulin. *J. Biol. Chem.* **267**, 5286–5295
28. Gao, Z.H., Krebs, J., VanBerkum, M.F.A., Tang, W., Maune, J.F., Means, A.R., Stull, J.T., and Beckingham, K. (1993) Activation of four enzymes by two series of calmodulin mutants with point mutations in individual Ca^{2+} binding sites. *J. Biol. Chem.* **268**, 20096–20104
29. Yazawa, M., Nakashima, K., and Yagi, K. A strange calmodulin of yeast. *Mol. Cell. Biochem.* **190**, 47–54
30. Yazawa, M., Ikura, M., Hikichi, K., Luan, Y., and Yagi, K. (1987) Communication between two globular domains of calmodulin in the presence of mastoparan or caldesmon fragment. *J. Biol. Chem.* **262**, 10951–10954
31. Kitagawa, C., Nakatomi, A., Hwang, D., Osaka, I., Fujimori, H., Kawasaki, H., Arakawa, R., Murakami, Y., and Ohki, S. (2011) Roles of the C-terminal residues of calmodulin in structure and function. *Biophysics* **7**, 35–49
32. Zhang, M., Li, M., Wang, J.H., and Vogel, H.J. (1994) The effect of Met Leu mutations on calmodulin's ability to activate cyclic nucleotide phosphodiesteras. *J. Biol. Chem.* **269**, 15546–15552
33. Wapinski, I., Pfeffer, A., Friedman, N., and Regev, A. (2007) Natural history and evolutionary principles of gene duplication in fungi. *Nature* **449**, 54–61
34. Wolfe, K. (2004) Evolutionary genomics: yeasts accelerate beyond BLAST. *Curr. Biol.* **14**, R392–R394
35. Fischer, R., Zekert, N., and Takeshita, N. (2008) Polarized growth in fungi: interplay between the cytoskeleton, positional markers and membrane domains. *Mol. Microbiol.* **68**, 813–826
36. Bauer, Y., Knechtle, P., Wendland, J., Helfer, H., and Philippsen, P. (2006) A Ras-like GTPase is involved in hyphal growth guidance in the filamentous fungus *Ashbya gossypii*. *Mol. Biol. Cell* **15**, 4622–4632