Importance of Phenylalanine Residues of Yeast Calmodulin for Target Binding and Activation*

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Recent genetic studies of yeast calmodulin (yCaM) have shown that alterations of different sets of Phe residues result in distinct functional defects (Ohya, Y., and Botstein, D. (1994) Science 263, 963–966). To examine the importance of Phe residues for target binding and activation, we purified mutant yCaMs containing single or double Phe to Ala substitutions and determined their ability to bind and activate two target proteins, calcineurin and CaM-dependent protein kinase (CaMK). Binding assays using the gel overlay technique and quantitative analyses using surface plasmon resonance measurements indicated that the binding of yCaM to calcineurin is impaired by either double mutations of F16A/F19A or a single mutation of F140A, while binding to CaMK is impaired by F89A, F92A, or F140A. These same mutant yCaMs fail to activate calcineurin and CaMK, respectively, in vitro. In addition, F19A exhibited a severe defect in activation of both enzymes. F12A activated calcineurin to only 50% of the level achieved by wild-type calmodulin but fully activated CaMK. These results suggest that each target protein requires a specific and distinct subset of Phe residues in yCaM for target binding and activation.

Calmodulin (CaM) is a ubiquitous eukaryotic Ca\(^{2+}\)-binding protein that regulates diverse cellular functions through its interaction with target proteins such as protein kinases, a protein phosphatase, ion channels, and cytoskeletal components. Strikingly, the CaM-binding motifs of these targets share a low degree of amino acid sequence homology to each other. This fact raises the question of how CaM recognizes such a diversity of target proteins. Structural studies have revealed that the binding of CaM to calcineurin is impaired by either double mutations of F16A/F19A or a single mutation of F140A, while binding to CaMK is impaired by F89A, F92A, or F140A. These same mutant yCaMs fail to activate calcineurin and CaMK, respectively, in vitro. In addition, F19A exhibited a severe defect in activation of both enzymes. F12A activated calcineurin to only 50% of the level achieved by wild-type calmodulin but fully activated CaMK. These results suggest that each target protein requires a specific and distinct subset of Phe residues in yCaM for target binding and activation.

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§ The abbreviations used are: CaM, calmodulin; yCaM, yeast calmodulin; CaMK, Ca\(^{2+}\)/calmodulin-dependent protein kinase; GST, glutathione S-transferase; SPR, surface plasmon resonance; HPLC, high pressure liquid chromatography; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

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calcineurin are encoded by CNA1/CNA2 and CNB1 (18–20), respectively, while CaMk is encoded by CMD1/CMK2 (21, 22). This study presents several lines of biochemical evidence indicating that Phe to Ala substitution results in altered target binding and activation and that the role of each Phe residue is different for calcineurin and CaMK.

**EXPERIMENTAL PROCEDURES**

*Media and Strains—*Media for growth of *S. cerevisiae* and *Escherichia coli* are as described (23). Genetic manipulation and yeast transformations were carried out as described (24). *S. cerevisiae* strains YCO202 [Ura3 leu2 lys2 trp1 ade2::CMD1::HIS3 (16) and DCNB1-A (MATa ade2 leu2 lys2 trp1 ade3::CMD1::HIS3)] were used as a positive and a negative control in halo assays, respectively. The other YOC strains have the same genetic background as YOC200 except that each strain harbors a cmd1 allele instead of CMD1 (16). *Escherichia coli* strain DH5α was used for propagation of plasmids and expression of recombinant proteins. *E. coli* strain BL21 carrying pLysS (25) was used for production of yCaMs, calcineurin, and CaMK.

*Materials—*Restriction enzymes and modifying enzymes were purchased from Takara (Kyoto, Japan) and New England Biolabs Inc. (Beverly, MA). Synthetic oligopeptides containing the yCaM-binding sequences of calcineurin and CaMK were expressed in *E. coli* strain JDH5α. Extracts containing individual fusion proteins were resolved by SDS-PAGE and proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated at room temperature for 1 h with 2% (v/v) nonfat dry milk in 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl and 0.05%Tween 20. The blocked membrane was probed at room temperature for 1 h with 400 nm or 4 μg biotinylated wild-type or mutant yCaM in CaCl2 buffer (10 mM Tris/HCl (pH 7.5), 0.2% bovine serum albumin, and 1 mM CaCl2). The membrane was washed with CaCl2 buffer three times, each for 10 min, and air-dried. Biotinylated horseradish peroxidase-avidin complex was coupled to biotinylated yCaM using Vectastain Elite standard kit (Vector laboratories). After washing with CaCl2 buffer three times, each for 10 min, the membrane was stained using the POD immunostain set (Wako).

*Surface Plasmon Resonance (SPR) Measurements—*Interactions of mutant yCaMs with Cna1p and Cmk1p were analyzed by SPR measurements using the IAasys biosensor (Fisons Applied Sensor Technology). All manipulations were carried out at 25°C. Cna1p and Cmk1p were covalently coupled to the carboxymethylxanthan-coated biosensor cuvette via the thiol group of the N-terminal cysteine residue according to the manufacturer’s instructions. After establishing a baseline with 20 μM Tris/HCl (pH 7.5) containing 1 mM CaCl2, 250 μM NaCl, and 0.05% Tween 20, the binding of mutant or wild-type yCaM was monitored as an increase in the evanescent field response. The cuvette was regenerated with 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA, 250 mM NaCl, and 0.05% Tween 20.

*Partial Purification of Recombinant Calcineurin—* *E. coli* strain BL21 carrying pLysS was transformed with each pET-cmd plasmid and in-

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1. Tanida, Y. Ohya, and Y. Anraku, unpublished result.

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CaCl2 and an appropriate concentration of ammonium sulfate (0.1–0.5 M) for each wild-type and mutant yCaM. yCaM was eluted with 10 mM Tris/HCl (pH 8.0) containing 1 mM EGTA and the same concentration of ammonium sulfate as used for washing. The eluate was concentrated by ultrafiltration using Centriprep 10, and stored at –20°C. HPLC gel filtration—HPLC gel filtration was performed using Tosk gel G3000SW (Toyoda-Soda) equilibrated with 50 mM Tris/HCl (pH 7.5) containing 200 mM NaCl and 1 mM CaCl2. Biotin Labeling of yCaM—Purified wild-type and mutant yCaMs were biotinylated using ImmunoPure Sulfo-NHS-LC-Biotin Kit (Pierce) according to the manufacturer’s instructions.

*Preparation of yCaMs—*Fusion proteins containing yCaM-binding sequences of calcineurin and CaMK were expressed in *E. coli* strain JDH5α. Extracts containing individual fusion proteins were resolved by SDS-PAGE and proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated at room temperature for 1 h with 2% (v/v) nonfat dry milk in 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl and 0.05% Tween 20. The blocked membrane was probed at room temperature for 1 h with 400 nm or 4 μg biotinylated wild-type or mutant yCaM in CaCl2 buffer (10 mM Tris/HCl (pH 7.5), 0.2% bovine serum albumin, and 1 mM CaCl2). The membrane was washed with CaCl2 buffer three times, each for 10 min, and air-dried. Biotinylated horseradish peroxidase-avidin complex was coupled to biotinylated yCaM using Vectastain Elite standard kit (Vector laboratories). After washing with CaCl2 buffer three times, each for 10 min, the membrane was stained using the POD immunostain set (Wako).

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*Partial Purification of Recombinant Calcineurin—* *E. coli* strain BL21 carrying pLysS was transformed with each pET-cna1 and pET-cnb1 and grown to an optical density at 600 nm of 0.5 at 23°C and of 0.25 at 37°C, respectively, in TB medium containing 100 μg/ml ampicillin and 25 μM/ml chloramphenicol. The production of Cna1p and Cnb1p was induced with isopropyl-1-thio-D-galactoside to final concentrations of 0.1 and 1 mM, respectively. Cells were harvested by centrifugation; resuspended in 50 mM Tris/HCl (pH 7.5) containing 10 mM EDTA, 10 mM DTT, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, and 5 μM/ml each of leupeptin, pepstatin A, aprotonin, and chymostatin); and then frozen-thawed. After sonication, the lysate was clarified by centrifugation at 100,000 × g for 1 h. Supernatants containing Cna1p and Cnb1p were mixed and concentrated by ultrafiltration and the purified protein was precipitated by adding 30% ammonium sulfate, and the precipitate was washed in buffer A (50 mM Tris/HCl (pH 7.5), 1 mM DTT, 1 mM CaCl2, and protease inhibitors) and was dialyzed against buffer A at 4°C. The dialysate was applied to yCaM-conjugated Sepharose 4B equilibrated with buffer A. After it was washed with 50 mM Tris/HCl (pH 7.5) containing 1 mM DTT, 1 mM CaCl2, 0.5 mM NaCl, and protease inhibitors, calcineurin was eluted with 50 mM Tris/HCl (pH 7.5) containing 1 mM DTT, 5 mM EGTA, and protease inhibitors. The eluate was dialyzed against 50 mM Tris/HCl (pH 7.5) containing 5 mM DTT, 5 mM sodium ascorbate, 0.5 mM FeSO4, and 50% glycerol.

*Preparation of yCaMs—*Fusion proteins containing yCaM-binding sequences of calcineurin and CaMK were expressed in *E. coli* strain JDH5α. Extracts containing individual fusion proteins were resolved by SDS-PAGE and proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated at room temperature for 1 h with 2% (v/v) nonfat dry milk in 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl and 0.05% Tween 20. The blocked membrane was probed at room temperature for 1 h with 400 nm or 4 μg biotinylated wild-type or mutant yCaM in CaCl2 buffer (10 mM Tris/HCl (pH 7.5), 0.2% bovine serum albumin, and 1 mM CaCl2). The membrane was washed with CaCl2 buffer three times, each for 10 min, and air-dried. Biotinylated horseradish peroxidase-avidin complex was coupled to biotinylated yCaM using Vectastain Elite standard kit (Vector laboratories). After washing with CaCl2 buffer three times, each for 10 min, the membrane was stained using the POD immunostain set (Wako).

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of the organic extraction procedure (28, 29). The reaction was terminated by adding 400 μl of 5 mm silicotungstate in 5 mM H2SO4 and 600 μl of a 1:1 mixture of isobutyl alcohol and benzene. After vortexing briefly, 80 μl of 5% (w/v) ammonium molybdate in 2 mM H2SO4 was added, and the solution was vigorously mixed for 10–15 s. The organic and inorganic phases were separated by centrifugation in a tabletop centrifuge for 5 min, and the amount of radioactivity in 400 μl of the organic phase was determined.

**Purification of Recombinant CaMK—**CaMK (Cmk1p) was purified as described previously with some modification (21). E. coli strain BL21 carrying pLySs was transformed with pET-CMK1 (21) and grown to an optical density at 600 nm of 0.1 at 37 °C in TB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Cmk1p production was induced by adding isopropyl-1-thio-β-D-galactoside to a final concentration of 0.5 mM. Cells were collected by centrifugation, resuspended in buffer C (20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 1 mM DTT, and protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, and 5 μg/ml each of leupeptin, pepstatin A, aprotonin, and chymostatin), and then freeze-thawed. After sonication, the lysate was clarified by centrifugation at 100,000 × g for 1 h. Nucleic acids were removed by adding 5% (w/v) proteamine sulfate followed by centrifugation at 100,000 × g for 20 min. The supernatant was adjusted to 0.2 M NaCl and applied to phenyl-Sepharose 6B equilibrated with buffer C containing 0.2 M NaCl. After washing with two bed volumes of buffer C containing 0.2 to 0 M NaCl in buffer C, the peak fractions of CaMK activity were pooled. Proteins in the pooled fractions were concentrated by 80% ammonium sulfate. The precipitate was resuspended in buffer D and dialyzed against buffer D (20 mM Tris/HCl (pH 7.5), 1 mM DTT, 1 mM CaCl2, 10% glycerol, and protease inhibitors) at 4 °C. The dialysate was applied to γCaM-conjugated Sepharose 4B equilibrated with buffer D. After washing with 20 mM Tris/HCl (pH 7.5) containing 1 mM DTT, 1 mM CaCl2, 0.5 M NaCl, and protease inhibitors, CaMK was eluted with 20 mM Tris/HCl (pH 7.5) containing 1 mM DTT, 5 mM EGTA, and protease inhibitors. The eluate was dialyzed against 20 mM Tris/HCl (pH 7.5) containing 1 mM DTT and 50% glycerol.

**Kinase Assay—**CaMK was assayed essentially as described previously (21) with some modifications. The reaction mixture (20 μl) containing 50 μM Tris/HCl (pH 7.5), 0.5 mg/ml bovine serum albumin, 1 mM CaCl2, 5 mM DTT, 10 mM MgCl2, 150 μM kemptoamide, 200 μM [γ-32P]ATP (specific activity approximately 100 cpm/pmol), γCaM, and CaMK was incubated at 30 °C for 30 min. The reaction was terminated by adding 10 μl of acetic acid. Incorporated Pi in kemptoamide was measured as described by Ohya et al. (21).

**Determination of Kinetic Parameters—**Dissociation constants from binding assays were determined as follows. First, the response change at equilibrium was determined by nonlinear regression with an equation representing one-site binding and plotted for each γCaM concentration. Dissociation constants were determined by nonlinear regression of this plot with the Michaelis-Menten equation. Kinetic parameters for enzyme activation were determined by nonlinear regression with the Michaelis-Menten equation.

**Halo Assays—**MA7a haploids were picked as colonies from fresh plates and grown at 23 °C in YPD medium. The fully grown culture was suspended in soft (1.5%) melted YPD agar that had been warmed at 55 °C to make 1–2 × 106 cells/ml of suspension. Cells were then plated on prewarmed YPD-Suc agar plates containing 1% Bacto-yeast extract, 2% polypeptone, 2% glucose, 2% sucinate and 2% agar. Glucose and sucinate were autoclaved separately. After solidification of the top agar, sterile filter disks (diameter 6 mm) were placed aseptically on the surface of the nascent lawn, and 1.6 nmol of synthetic α-factor (5 μl) was pipetted onto sterile filter disks. The plates were incubated at 23 °C for 3, 5, and 7 days before photographs were taken. Because of the extended period of incubation, this assay has been useful to measure the capacity of cells to recover from α-factor arrest and resume growth. These assays were performed for each γCaM mutant at least three times.

**RESULTS**

**SDS-PAGE and Gel Filtration Chromatography of Mutant γCaMs—**All of the individual Phe to Ala mutants of γCaM as well as the double mutants (F16A/F19A and F65A/F68A) could be expressed at a reasonably high level in E. coli and purified by Ca2+-dependent phenyl-Sepharose chromatography. It is known that γCaM, like mammalian CaM, migrates differently during SDS-PAGE in the presence of Cu2+ or EGTA (27). The presence of Cu2+ increases the electrophoretic mobility of γCaM. Fig. 1 shows the SDS-PAGE results for wild-type γCaM and γCaMs harboring a single point mutation (F12A, F16A, F19A, F89A, F92A, or F140A) or double mutations (F65A/F68A). Wild-type γCaM and all of the single-point mutant γCaMs exhibited an apparent molecular mass of 16.0 ± 0.2 kDa in the presence of EGTA and 12.6 ± 0.2 kDa in the presence of Ca2+ during SDS-PAGE (Fig. 1). In contrast, some of the mutants with two or more Phe to Ala substitutions showed significantly altered mobility in the presence of EGTA. For example, F65A/F68A exhibited a larger apparent molecular mass of 16.8 ± 0.02 kDa in the presence of EGTA (21). However, another double mutant, F16A/F19A, showed a mobility similar to that of wild-type γCaM (data not shown), indicating that double mutations do not always result in the irregular mobility. Thus, the single substitution Phe to Ala mutations and the F16A/F19A double mutation combination did not significantly perturb the global protein structure of γCaM. A triple mutant protein, F12A/F16A/F19A ran more slowly than wild-type γCaM, exhibiting an apparent molecular mass of 17.1 kDa in the presence of EGTA and 13.2 kDa in the presence of Ca2+. A quintuple mutant protein, F12A/F16A/F19A/F65A/F68A, that caused a lethal effect in yeast cells ran even more slowly (data not shown). Thus, the altered mobility of the triple and quintuple mutant proteins is probably due to global conformational changes induced by the multiple Phe to Ala substitutions. For the purpose of examining the global conformations of mutant CaMs under native conditions, gel filtration chromatography

![Fig. 1. SDS-PAGE of mutant γCaMs. The proteins were run on a 15% SDS-polyacrylamide gel in the presence of 5 mM EGTA (A) or 1 mM CaCl2 (B). Lane 1, wild type; lane 2, F12A; lane 3, F16A; lane 4, F19A; lane 5, F65A/F68A; lane 6, F89A; lane 7, F92A; lane 8, F140A.](Image 324x370 to 538x729)
can be used (14). All of the single substitution mutant yCaMs exhibited nearly identical retention times, further suggesting that the single substitution mutations do not cause global structural perturbations of yCaM (Table I).

**Gel Overlay Assays of Calcineurin and CaMK with Mutant yCaMs**—We performed gel overlay assays to investigate the allele-specific binding of mutant yCaMs to calcineurin and CaMK. Fragments of the target enzymes containing the yCaM-binding sequences were expressed in *E. coli* as fusions with GST for Cna1p, Cmk1p, and Cmk2p or as a fusion with MalE for Cna2p (Fig. 2A). Fig. 2B clearly shows that the binding of mutant yCaMs to each target protein is allele-specific and that binding specificity differs for calcineurin and CaMK. yCaM binding to GST-Cna1 and MalE-Cna2 were severely impaired for mutant yCaMs possessing the substitutions F19A or F140A (lanes 4, 5, 8, and 9). On the other hand, yCaM binding to GST-Cmk1 or GST-Cmk2 was not detected for mutant yCaMs possessing a mutation in the C-terminal half, F89A, F92A, or F140A (lanes 6–8). A triple mutant protein, F12A/F16A/F19A, also failed to bind to both GST-Cmk1 and GST-Cmk2. For each yCaM, equivalent binding was always observed to the two different isoforms of calcineurin, GST-Cna1 and MalE-Cna2.

### Table I

| Allele         | Retention time (min) |
|----------------|----------------------|
| Wild type      | 18.5                 |
| F12A           | 18.4                 |
| F16A           | 18.5                 |
| F19A           | 18.5                 |
| F65A/F68A      | 18.5                 |
| F89A           | 18.5                 |
| F92A           | 18.4                 |
| F140A          | 18.3                 |

For binding to Cna1pep, F16A/F19A and F140A, both of which showed no detectable binding activity to GST-Cna1 in the gel overlay assay, exhibited dissociation constants that were more than 10-fold larger than that of wild-type yCaM. In contrast, F19A, which showed severely decreased binding to GST-Cna1 in the gel overlay assay, exhibited only a slightly increased dissociation constant. F12A, F16A, and F65A/F68A exhibited dissociation constants that were similar to that of wild-type yCaM. These observations are consistent with the finding that these yCaMs showed no defect in yCaM binding in the gel overlay assay.

For binding to Cmk1pep, the responses of F89A, F92A, and F140A, all of which failed to bind to GST-Cmk1, were too small to determine the dissociation constants, indicating that the affinities of these mutant yCaMs to Cmk1pep are considerably decreased. The dissociation constants of F12A, F16A, F19A, and F65A/F68A are similar to (and that of F16A/F19A is slightly larger than) that of wild-type yCaM. These results are consistent with the findings from the gel overlay assay.

**Activation of Calcineurin and CaMK by Mutant yCaMs**—The functional effects of Phe to Ala substitutions were further stud-
under "Experimental Procedures." Maximal activity was determined by nonlinear regression as described under "Experimental Procedures." $K_d$ is the concentration of yCaM that produces half-maximal binding level. Maximal binding level and $K_d$ were determined by nonlinear regression as described under "Experimental Procedures." $K_{CaM}$ is the concentration of yCaM that produces half-maximal activation and was determined by nonlinear regression as described under "Experimental Procedures." $V_{max}$ is the maximal observed activity, expressed as the percentage of the maximal activity observed for wild-type yCaM. Maximal activity was determined by nonlinear regression as described under "Experimental Procedures."

| yCaM     | Calciumin        | CaMK              |
|----------|------------------|-------------------|
|          | $K_d$  | $K_{CaM}$ | $V_{max}$ | $K_d$  | $K_{CaM}$ | $V_{max}$ |
| Wild type| 57     | 61       | 100%     | 0.72   | 0.40     | 100%     |
| F12A     | 36     | 100      | 54%      | 0.42   | 1.7       | 101%     |
| F16A     | 94     | 170      | 102%     | 1.2    | 2.1       | 77%      |
| F19A     | 170    | 130      | 13%      | 0.99   | 1.1       | 30%      |
| F16A/F19A| 790    | 630      | 20%      | 1.9    | ND        | ND       |
| F65A/F68A| 65     | 81       | 64%      | 0.36   | 1.5       | 90%      |
| F89A     | 190    | 98       | 86%      | ND     | ND        | ND       |
| F92A     | 310    | 160      | 89%      | ND     | ND        | ND       |
| F140A    | 820    | 230      | 59%      | ND     | ND        | ND       |

$^a$ 0.62 nmol of P/mg/min.
$^b$ 54 nmol of P/mg/min.
$^c$ ND, not determined.

Table II
Kinetic parameters for binding and activation of enzymes by mutant yCaMs

...mated pheromone. As expected from the biochemical results for calciumin, yeast strains expressing the mutant yCaM harboring the F16A/F19A substitutions showed clear halo phenotypes like $\Delta cnb1$ (Fig. 5). A strain expressing F12A/F140A showed an intermediate phenotype (Fig. 5). All other mutants except strains expressing F12A/F92A showed turbid halo phenotypes (Table III). In vitro, we observed that F12A/F92A can activate calciumin (data not shown); therefore, the mutations of F12A/F92A might impair another yCaM regulatory pathway other than calciumin that is essential for adaptation to mating pheromone. The clear halo phenotypes we observed did not simply reflect the slower growth of the mutants, because prolonged incubation for more than a week did not change the phenotype.

**DISCUSSION**

In this study, we examined the ability of mutant yCaMs containing Phe to Ala substitutions to bind and activate two target proteins using a gel overlay assay, SPR measurements, enzymatic assays, and genetic analyses. Our results clearly...
indicate that a distinct set of Phe residues of yCaM is required to bind and activate each target enzyme.

The binding of mutant yCaMs to calcineurin and CaMK was examined by three independent binding assays: gel overlay assay, SPR measurements, and the assay of enzyme activation. The inability of F16A/F19A to bind to the GST-Cna1 and MalE-Cna2 fusions on a membrane and the significantly larger dissociation and activation constants of F16A/F19A for calcineurin indicate that Phe-16 and Phe-19 of yCaM are required for this interaction. Phe-19 seems slightly more important for the binding than Phe-16, since the single substitution mutation of F19A itself has a more profound effect as determined by gel overlay assay and SPR measurements. In addition, Phe-140 is also important for the interaction with calcineurin based on these binding assays. In contrast, binding to CaMK is markedly diminished by the C-terminal mutations of F89A, F92A, or F140A. The importance of the C-terminal Phe residues for CaMK binding suggests that the C-terminal domain of yCaM dominantly contributes to the whole binding energy of the yCaM-CaMK complex. Although the binding of F12A/F16A/F19A to CaMK was also not detected by gel overlay assay, this finding may be due to structural perturbations caused by the multiple mutations (see discussion below).

The dissociation and activation constants of F19A are similar to those of F89A and F92A, both of which could bind to the fusions of calcineurin on a membrane. However, gel overlay assays showed that F19A failed to bind to fusion proteins of calcineurin on a membrane. This discrepancy might arise from the denatured forms of the fusion proteins used in gel overlay assays. We assume that F19A has the ability to bind to native calcineurin but fails to bind to denatured calcineurin. Alternat-

| Allele   | Phenotype |
|----------|-----------|
| Wild type| +         |
| Δcnb1    | -         |
| F12A     | +         |
| F16A/F19A| -         |
| F65A/F68A| +         |
| F89A     | +         |
| F92A     | +         |
| F140A    | +         |
| F12A/F89A| +         |
| F12A/F92A| -         |
| F12A/F140A| ±      |
| F12A/F16A/F19A| - |

**FIG. 4.** Activation of CaMK by mutant yCaMs. The activity of CaMK was measured at different concentrations of yCaM as described under “Experimental Procedures.” Maximal activity (100%) was set at the Vmax value of wild-type yCaM obtained from nonlinear regression of the Michaelis-Menten equation (Table II). A, N-terminal mutant yCaMs; B, C-terminal mutant yCaMs.

**FIG. 5.** Halo assays of yCaM mutants. MATα strains were exposed to 1.6 nmol of synthetic α-factor at 23 °C in YPD top agar. Halos were photographed after 5 days of incubation.

**Table III**

Halo assays of yCaM mutants

The phenotypes of yCaM mutants are indicated as follows: +, a turbid halo phenotype; −, a less cloudy halo phenotype; ±, a clear halo phenotype.
tively, F19A might have a smaller association rate constant or a larger dissociation rate constant for calcineurin, although we think this is unlikely. The association rate constants of F19A and F92A were determined to be $1.1 \times 10^6$ M$^{-1}$ s$^{-1}$ and $1.4 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively, by SPR measurements (data not shown). Accordingly, the calculated dissociation rate constants of F19A and F92A were 0.19 s$^{-1}$ and 0.44 s$^{-1}$, respectively.

The activation and dissociation constants of F140A were different for calcineurin. This discrepancy might result from the immobilization of the N terminus of Cna1pep in the binding assay. For optimal binding, F140A might position its C-terminal lobe slightly differently from that of wild-type relative to the yCaM-binding domain of calcineurin. If the C-terminal lobe of yCaM binds to the N terminus of the yCaM-binding domain of calcineurin as observed in the three structures of CaM-peptide complexes, such alteration in the positioning of the C-terminal lobe of F140A might be sterically hindered in binding to Cna1pep with its N terminus immobilized on carboxymethylated dextran.

The ability of each mutant yCaM to activate target enzymes was assessed by comparing the maximal activation achieved by each mutant yCaM relative to wild-type yCaM. Calcineurin is hardly activated by F19A and F16A/F19A. F12A and F140A also exhibit some defects in activation of calcineurin. Consistent with these results, yeast strains expressing only F16A/F19A, F12A/F16A/F19A, or F12A/F140A showed the same phenotypes as that of a calcineurin-deficient strain, suggesting that these mutant yCaMs cannot stimulate calcineurin in vivo to the level required to display the wild-type phenotype. CaMK is activated only slightly by F19A and F16A/F19A as well as F89A, F92A, and F140A. The inability of F89A, F92A, and F140A to activate CaMK may be due to defective binding to CaMK at the concentrations of yCaM used in the assay. A specific role for F19A in CaMK activation is suggested, since F19A retains most of its binding ability.

We examined whether Phe to Ala mutations in yCaM affect its overall structure and Ca$^{2+}$-dependent conformational change. The mutations of a single Phe residue and the double substitution F16A/F19A in yCaM do not have significant effects on the global protein structure as judged by their interaction with phenyl-Sepharose, their mobility during SDS-PAGE in the presence of Ca$^{2+}$ or EGTA, and their retention time in gel filtration chromatography in the presence of Ca$^{2+}$. Although F65A/F68A exhibits a slower electrophoretic mobility than wild-type yCaM in the presence of EGTA (apparent molecular mass of 16.9 kDa), it shows the same mobility as wild type in the presence of Ca$^{2+}$, suggesting that Ca$^{2+}$ binding restores the native conformation. This may explain why F65A/F68A has the ability to bind to both target proteins well. In contrast, some yCaMs containing multiple Phe to Ala substitutions showed aberrant mobility in the presence of EGTA or Ca$^{2+}$ (16). A triplet mutant protein, F12A/F16A/F19A, and a quintuple mutant protein, F12A/F16A/F19A/F65A/F68A, both show a much slower electrophoretic mobility than wild-type yCaM. Multiple replacements of Phe by Ala could cause a considerable gap in the hydrophobic core of yCaM, thereby destabilizing the native conformation of yCaM. That normal overall protein structure is preserved in single substitution mutant proteins is further suggested by the in vivo phenotypes of yCaM mutants. Yeast strains expressing each single-substitution mutant yCaM are all viable at 30°C (16). This indicates that these mutant yCaMs can interact with all of the targets that are essential for cell proliferation at 30°C. Taken together, all of the single mutant yCaMs as well as F16A/F19A and F65A/F68A have the same global conformation as wild-type yCaM, at least in the presence of Ca$^{2+}$.

Subsequent to our analyses of the functional importance of Phe residues in yCaM, a mutant of human CaM containing the F92A substitution was analyzed (32). In contrast to the F92A mutant yCaM, the F92A mutant human CaM displays a major defect in Ca$^{2+}$-induced conformational transition. First, the F92A human CaM runs significantly more slowly than wild-type CaM during gel electrophoresis (32), while the F92A mutant yCaM is indistinguishable from wild-type yCaM in mobility (Fig. 1). Second, the Ca$^{2+}$-dependent binding of hydrophobic probes to the F92A human CaM is lower than that of wild-type human CaM, while the Ca$^{2+}$-dependent binding of the hydrophobic probes to the F92A yCaM and wild-type yCaM is very similar (data not shown). These results suggest that the F92A substitution causes much less of a change in the exposure of hydrophobic surfaces in yCaM than it does in human CaM.

yCaM mutants harboring one or several Phe to Ala mutations were classified into four intragenic complementation groups (17). Each group showed a different characteristic functional defect in actin organization, yCaM localization, nuclear division, or bud emergence. Based on analyses of the complementing yCaM mutants, we have proposed that each complementation group is specifically defective for the activation of one essential target of yCaM (17). This study has revealed that distinct subsets of Phe residues in yCaM are in fact required for activation of calcineurin and CaMK, thus providing a biochemical basis for the observed complementation. Phe-92 of yCaM has recently been shown to be essential for the interaction with Myo2p (33), one of the essential calmodulin targets in S. cerevisiae (34). Thus, given that Myo2p is biochemically assigned to one of the intragenic complementation groups, Phe residue(s) other than Phe-92 must participate in the regulation of another known essential target, Nuf1p/Sqc110p (35, 36). Furthermore, yCaM binding to the other unknown essential targets must also require specific Phe residues.

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