

Calcium Binding Is Required for Calmodulin Function in *Aspergillus nidulans*

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To explore the structural basis for the essential role of calmodulin (CaM) in *Aspergillus nidulans*, we have compared the biochemical and in vivo properties of *A. nidulans* CaM (AnCaM) with those of heterologous CaMs. Neither *Saccharomyces cerevisiae* CaM (ScCaM) nor a Ca²⁺ binding mutant of *A. nidulans* CaM (1234) interacts appreciably with *A. nidulans* CaM binding proteins by an overlay assay or activates two essential CaMKs, CMKA and CMKB. In contrast, although vertebrate CaM (VCaM) binds a spectrum of proteins similar to that for AnCaM, it is unable to fully activate CMKA and CMKB, displaying a higher K_{CaM} and reduced V_{max} for both enzymes. In correlation with the biochemical analysis, neither ScCaM nor 1234 can support *A. nidulans* growth in the absence of the endogenous protein, whereas VCaM only partially complements the absence of wild-type CaM. Analysis of VCaM and AnCaM chimeras demonstrates that amino acid variations in both N- and C-terminal domains contribute to the inability of VCaM to activate CMKB, but differences in the N terminus are largely responsible for the reduced activity towards CMKA. In vivo, the chimeric molecules support growth equivalently, but only to levels intermediate between those of VCaM and AnCaM, suggesting that the reduced ability to activate the CaMKs is not solely responsible for the inability of VCaM to complement the absence of the wild-type protein. Thus, not only is Ca²⁺ binding required for CaM function in *A. nidulans*, but the essential in vivo functions of *A. nidulans* CaM are uniquely sensitive to the subtle amino acid variations present in vertebrate CaM.

Calmodulin (CaM), an EF-hand Ca²⁺ binding protein, is highly conserved throughout evolution, with the vertebrate CaMs being identical at the amino acid level (2). In *Saccharomyces cerevisiae* (6), *Schizosaccharomyces pombe* (44), *Aspergillus nidulans* (40), and *Drosophila melanogaster* (14), the unique CaM gene is essential. CaM regulates its binding partners in both Ca²⁺-dependent and Ca²⁺-independent manners (2, 17). Genetic studies suggest that Ca²⁺/CaM-dependent pathways are essential for the growth of fission yeast but not of budding yeast (27). In *S. pombe*, CaM requires at least one functional Ca²⁺ binding site to support growth (30). However, the *S. cerevisiae* CaM protein contains only three functional EF-hand motifs rather than the four Ca²⁺ binding sites found in all other CaMs and, in contrast to results of complementation studies with *S. pombe* (30), does not require Ca²⁺ binding to support *S. cerevisiae* growth (13). However, in both budding and fission yeast, vertebrate CaM complements the yeast null strains, suggesting that vertebrate CaM can perform both Ca²⁺-dependent and Ca²⁺-independent essential functions in vivo (5, 30, 34).

For *S. cerevisiae*, all identified CaM binding proteins that are essential for growth under normal circumstances do not require Ca²⁺ binding, consistent with the importance of the Ca²⁺-independent roles of CaM in this organism. However, several critical functions for Ca²⁺-dependent CaM targets, calcineurin and CaM kinases, have been identified for *S. cerevisiae*, including survival of pheromone-induced growth arrest (3,

4, 29, 45), salt tolerance (1, 12, 15, 28, 31), and acquired thermotolerance (18). In *S. pombe*, no Ca²⁺-independent targets of CaM essential for growth have been identified. Whereas calcineurin is not essential for growth (47), other Ca²⁺/CaM-dependent enzymes, such as the serine/threonine kinase CMK, have been identified but not evaluated in the context of gene deletion studies. Thus, neither *S. pombe* nor *S. cerevisiae* provides a system in which Ca²⁺-regulated CaM structure-function relationships can be studied in vitro and in vivo in the context of known essential Ca²⁺/CaM-dependent targets.

In contrast to the yeasts, other fungi such as *A. nidulans* and *Cryptococcus neoformans* do contain genes encoding Ca²⁺/CaM binding proteins that are essential for growth. In *C. neoformans*, the fungal pathogen that causes meningitis in immunocompromised patients, both calcineurin A and B genes are required for growth at 37°C and for virulence (11, 33). The filamentous fungus *A. nidulans* possesses both Ca²⁺-dependent and Ca²⁺-independent targets of CaM that are essential for growth. In *A. nidulans*, four Ca²⁺/CaM-dependent enzymes, consisting of three serine/threonine protein kinases and one phosphatase (calcineurin), are homologous to known mammalian enzymes, and three of the four are essential. The protein kinases CMKA/CMPK and CMKB are required for the G₂-to-M transition and for the reentry of spores into the proliferative cycle, respectively (7, 20), whereas calcineurin is required in G₁ (39, 32). On the other hand, *A. nidulans* also possesses Ca²⁺-independent targets of CaM, such as the class I myosin MyoA, that are essential for growth (24).

We have examined structural requirements of *A. nidulans* CaM both in vitro and in vivo. First, we demonstrate that Ca²⁺ binding is essential for the activation of CMKA and CMKB

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TABLE 1. *A. nidulans* strains used in this study

Strain	Genotype	Source
GR5	A773; <i>pyr</i> G89; <i>w</i> A2; <i>pyro</i> A4	Rasmussen et al. (40)
JJ10	A773; <i>w</i> A2	This study
AlcCaM	A773; <i>alc:cam</i> ; <i>w</i> A2; <i>pyro</i> A4	Lu et al. (23)
JJ12	A773; <i>alc:cam</i> ; <i>w</i> A2	This study
JJ13	A773; <i>alc:cam</i> ; <i>gpd:cam</i> ; <i>w</i> A2	This study
JJ14	A773; <i>alc:cam</i> ; <i>gpd:cmd</i> 1; <i>w</i> A2	This study
JJ15	A773; <i>alc:cam</i> ; <i>gpd:vCaM</i> ; <i>w</i> A2	This study
JJ16	A773; <i>alc:cam</i> ; <i>gpd:cam</i> 1234; <i>w</i> A2	This study
JJ17	A773; <i>alc:cam</i> ; <i>gpd:cam</i> ANVC; <i>w</i> A2	This study
JJ18	A773; <i>alc:cam</i> ; <i>gpd:cam</i> VNAC; <i>w</i> A2	This study

and is required to support growth in *A. nidulans*. Although vertebrate CaM appears to bind most CaM binding proteins in *A. nidulans* extracts, it is unable to fully activate CMKA and CMKB and only partially supports growth in the absence of endogenous CaM. Analysis of chimeras between *A. nidulans* and vertebrate CaM demonstrates that the N-terminal domain of VCaM harbors variations resulting in a severe defect in the activation of CMKA. However, neither chimeric CaM fully complements the loss of endogenous CaM in *A. nidulans*. These results demonstrate that while the CaM amino acid sequence is highly conserved, the protein does possess subtle sequence and structural variations that can dramatically alter the essential functions of the molecule in an organism-specific manner.

MATERIALS AND METHODS

Medium and growth conditions. *A. nidulans* conidia were grown in minimal medium containing 50 mM glycerol (MMG), 50 mM glycerol plus 100 mM Thr (MMGT), or 50 mM glucose (MMD) as the sole carbon source as described by Lu and Means (22). For strains with the *pyr*G89 or *pyro*A4 phenotype, the medium was supplemented with 5 mM uridine and 10 mM uracil or 2 μ g of pyridoxine hydrochloride/liter, respectively. All strains were propagated at 37°C.

Generation of CaM expression vectors. A multistep process was used to generate a CaM expression plasmid containing the *gpdA* promoter, the *pyroA* nutritional marker, and the *trpC* terminator sequence. First, a 1.42-kb *Xba*I/*Nco*I fragment of pAN 7-1 was ligated into a 4.75-kb *Xba*I/*Nco*I fragment of pAN 8-1 (both pAN 7-1 and 8-1 were gifts of A. M. J. J. Van Den Hondel [36]) to generate pAN 8-7. Second, pAN 8-7 was digested with *Xba*I, blunt ended, and digested with *Eco*RI. The resulting 3.22-kb fragment, containing the *gpdA* promoter and *trpC* terminator, was subcloned into the *Eco*RI and blunted *Pst*I sites of pSX, pUC18 containing the 2.5-kb *pyroA* nutritional marker, thus generating pSXG. All CaMs were expressed as hemagglutinin (HA)-tagged fusion proteins to facilitate detection. The HA epitope was incorporated into the *A. nidulans* CaM cDNA by PCR, and the PCR product was cloned into *Nco*I/*Bam*HI-digested pSXG as an *Afl*III/*Bam*HI fragment generating pSXG-AnCaM. The *S. cerevisiae* (gift of T. Davis) cDNA and all *A. nidulans* mutant cDNAs were subcloned into the *Nco*I/*Bam*HI-digested pSXG-AnCaM. The chicken CaM cDNA was subcloned into the *Nco*I and blunted *Bam*HI sites as an *Nco*I/blunted *Xba*I fragment from pCaMpl (37). All point mutations and chimeric CaM cDNAs were generated using the megaprimer PCR-based mutagenesis technique (42). All CaM cDNAs were sequenced prior to use.

Ectopic CaM expression in *A. nidulans*. *A. nidulans* strains used in this study are listed in Table 1. The AlcCaM strain of *A. nidulans* was transformed with the CaM expression vectors by either electroporation (41) or conventional polyethylene glycol-mediated transformation (22). The control strains were generated by transforming the GR5 strain with pAL5 and pSXG vectors and the AlcCaM strain with pSXG. Positive transformants were screened for ectopic CaM protein expression. Extracts were generated from spores germinated for 15 h in MMG. Following filtration, the mycelia were frozen in liquid N₂. The frozen mycelia were ground in a cold mortar and pestle in protein extraction buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 10 mM benzimidazole, 1 μ g of pepstatin A/ml, 1 μ g of leupeptin/ml and 1 μ g of aprotinin/ml). The protein extracts were clarified by

centrifugation at 16,000 \times g for 10 min at 4°C. Western analysis was performed using 100 μ g of crude protein extract, the 12CA5 anti-HA antibody (Boehringer-Mannheim, Indianapolis, Ind.), goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G (Jackson Laboratory, Bar Harbor, Maine), and the Enhanced Chemiluminescence kit (Amersham, Piscataway, N.J.).

CaM quantification. The concentrations of endogenous, ectopically expressed *A. nidulans* CaM and the four-Ca²⁺-binding-site mutant CaM were determined by radioimmunoassay (RIA), as described by Rasmussen et al. (40). Semiquantitative analysis of *S. cerevisiae* and chicken CaM expression was performed by comparative Western blot analysis, comparing crude extracts from strains to amino acid analyzed standards using a polyclonal anti-cmd1 antibody (gift of T. Davis) and a monoclonal anti-CaM antibody (UBI, Lake Placid, N.Y.), respectively. The relative expression levels of the chimeric CaMs were determined by direct comparison to the AnCaM expressing strain by Western analysis using a monoclonal 12CA5 anti-HA antibody.

***A. nidulans* growth.** The ability of the ectopically expressed CaMs to functionally complement the absence of wild-type CaM was determined by a colony diameter assay as described by McGoldrick et al. (24). Briefly, the spores were diluted and plated on either MMG or MMD agar plates at a concentration of approximately four spores per plate. The average colony diameter was measured every 24 h for 5 days.

Calmodulin overlay. CaM overlay was performed using protein A-CaM fusion proteins as described by Stirling et al. (43). The protein A-CaM fusion proteins were generated in a multistep process. First, pALP1 (gift of M. Stark) was digested with *Eco*RI, blunted, and digested with *Bam*HI. A HA-tagged *A. nidulans* CaM cDNA PCR product was subcloned as a blunted *Afl*III/*Bam*HI fragment, generating pALP-AnCaM. To place the chicken CaM and the four-Ca²⁺-binding-site mutant CaM into the pALP1 vector as HA-tagged protein A CaM fusion proteins, the cDNAs were first shuttled into the blunted *Eco*RI/*Bam*HI sites of the original pALP1 plasmid as *Pvu*II/*Bam*HI fragments from the pSXG-based expression plasmids described above. Finally, the cDNAs were interchanged with the AnCaM cDNA in pALP-AnCaM as *Nco*I/*Bam*HI fragments, thereby creating pALP-VCaM and pALP-1234. The *S. cerevisiae* CaM-protein A fusion protein was expressed using pDS100 that was kindly provided by M. Stark (43). The fusion proteins were expressed constitutively in BL-21 bacteria and purified on phenyl-Sepharose resin (38). The concentration of the CaM fusion protein used in the overlay medium was 175 nM (6 μ g/ml).

CaM kinase purification and assays. The CaM concentrations required for half-maximal kinase activation (K_{CaM}) of both CMKA and CMKB were determined using bacterially expressed hexa-histidine-tagged protein kinases and bacterially expressed CaM. Both CMKA and CMKB were subcloned into pTrcHisB (Invitrogen, Carlsbad, Calif.) independently, CMKA as a *Sac*I/*Eco*RI fragment and CMKB as a *Bam*HI/*Eco*RI fragment. The kinases were expressed and purified as described by Joseph and Means (20). Kinase assays were performed in 30- μ l reaction mixtures in a buffer containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol, 0.1% Tween 20, 200 μ M ATP, 0.2 μ l of [γ -³²P]ATP/reaction, and a 200 μ M concentration of the peptide substrate ADR1G (LKKLTRRASFSGQ) (8). CaM was added to reactions at various concentrations up to 1 μ M, and the reactions were initiated with the addition of either 200 or 400 ng of CMKB and incubated at 30°C for either 5 or 10 min for CMKA and CMKB, respectively. The reactions were terminated by transferring 20 μ l of the reaction mixture onto p81 phosphocellulose filters (Millipore, Bedford, Mass.), followed by extensive washing in 75 mM phosphoric acid (9). The specific activity was determined following liquid scintillation counting of the dried filters.

RESULTS

Vertebrate CaM is unable to fully activate CMKA and CMKB. We first compared the abilities of *A. nidulans* CaM (AnCaM), *S. cerevisiae* CaM (ScCaM), vertebrate CaM (VCaM), and a Ca²⁺ binding mutant of *A. nidulans* CaM (1234) to interact with *A. nidulans* CaM binding proteins in an overlay assay. AnCaM is highly homologous with VCaM and ScCaM, sharing 84 and 60% amino acid identity, respectively (Fig. 1). Although the sequence is highly conserved, the abilities of these CaMs to bind proteins from crude *A. nidulans* extracts vary remarkably. AnCaM and VCaM bind similar spectra of proteins by CaM overlay, including two abundant and essential Ca²⁺/CaM-dependent enzymes, CNA and

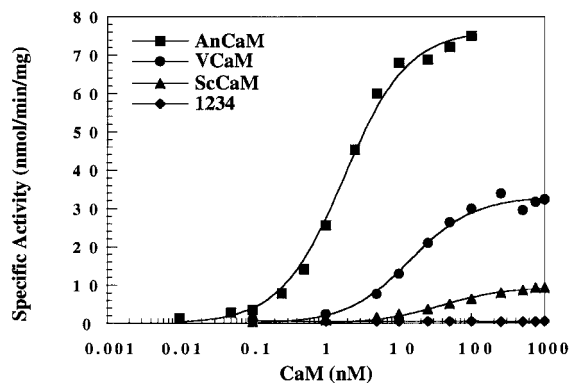
<i>A. nidulans</i> CaM	1	MADSLTTEEQVSEYKEAFSLFDKDG	QITTKELGTVMRS LGQNPSESELDQDMINEVDADN
Vertebrate CaM	1	MADQLTTEEQIAEFKEAFSLFDKDG	GIITTKELGTVMRS LGQNPTEAEIQDMINEVDADG
<i>S. cerevisiae</i> CaM	1	MSSNLTTEEQIAEFKEAFALFDKDN	GSFSSSELATVMRS LGLSPSSEAEVNDLMMNEIDVDG
		++	*
<i>A. nidulans</i> CaM	61	NGTIDFPFELTMMARKMKD TDSEEEIREAFKVFDR	DNNGFI SA AELRHVMTSIGEKLTD
Vertebrate CaM	61	NGTIDFPFELTMMARKMKD TDSEEEIREAFRVFDKDG	NGYI SA AELRHVMTNIGEKLTD
<i>S. cerevisiae</i> CaM	61	NHQLEFSEFLALMSRQLKSN	DS EQELLEAFKVFDKMGDGLI SA AELKRVLT SIGEKLTD
			*
<i>A. nidulans</i> CaM	121	EVDEMIREADQ	DGDGRITDYNEFVQLMMOK
Vertebrate CaM	121	EVDEMIREADI	DGDGQVNYE EEFVQMTAK
<i>S. cerevisiae</i> CaM	121	EVDDMLREVS-	DGSGEINIQQEAALSK-

FIG. 1. Alignment of *A. nidulans* CaM with vertebrate and *S. cerevisiae* CaMs. The *A. nidulans* (40) (J05545), vertebrate (M36167), and *S. cerevisiae* (6) (P06787) CaM sequences were aligned using FASTA and shaded by BOXSHADE. Asterisks indicate the Glu residues mutated to Ala to generate the Ca²⁺-binding mutant CaM. The junction of the *A. nidulans*/vertebrate chimeric CaMs lies between the amino acids marked with pluses.

CMKA (Fig. 2). In contrast, ScCaM binds few, if any, proteins and displays a pattern similar to that of the Ca²⁺ binding mutant of AnCaM (1234), in which the Glu residue at position 12 of each EF hand is mutated to Ala (Fig. 1). Ye and Bretscher (46) used a similar overlay assay to show that *S. cerevisiae* CaM also fails to bind the CaM binding proteins present in bovine brain.

To further investigate the differential functions of these heterologous CaM proteins, we tested their ability to activate two essential CaMKs present in *A. nidulans*, CMKA and CMKB (Fig. 3 and Table 2). Consistent with the CaM overlay, neither ScCaM nor 1234 is able to appreciably activate these kinases under the assay conditions. However, VCaM is unable to fully activate these kinases despite its similarity to AnCaM in the CaM overlay assay. The most profound difference between

A.



B.

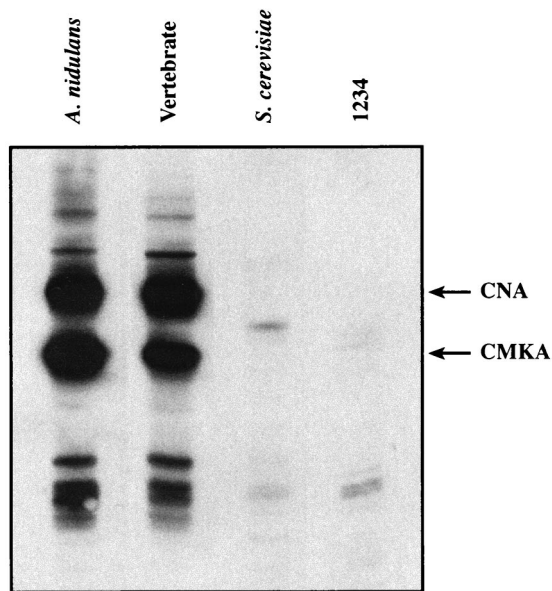
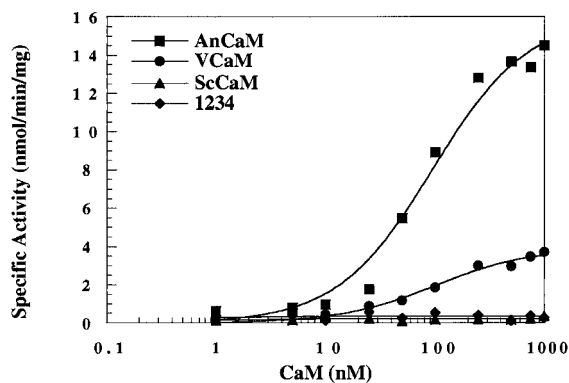


FIG. 2. Identification of *A. nidulans* CaM binding proteins by overlay assay. *A. nidulans* crude protein extract was separated by polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane, and probed with either *A. nidulans* CaM, vertebrate CaM, *S. cerevisiae* CaM, or the Ca²⁺ binding mutant CaM-protein A fusion protein. The blots are identical exposures of 100 µg of crude protein extract probed in the presence of Ca²⁺ as described in Materials and Methods.

FIG. 3. Activation of CMKA and CMKB by *A. nidulans* and heterologous CaMs. The specific activity of hexa-histidine-tagged bacterially expressed and purified CMKA (A) and CMKB (B) was measured at different concentrations of purified AnCaM (closed square), VCaM (closed circle), ScCaM (closed triangle), or 1234 (closed diamond) CaM as described in Materials and Methods. The graphs are representative of three independent experiments.

TABLE 2. CaM expression in medium containing glycerol or glucose

Strain	Amt of endogenous CaM ^a (ng/mg)		Amt of heterologous CaM ^b (ng/mg)
	Glycerol	Glucose	Glucose
GR5	256	193	NA ^c
AlcCaM	40	<2	NA
AlcCaM/VCaM	ND ^d	ND	233
AlcCaM/ScCaM	24	<2	158
AlcCaM/1234	ND	ND	1,418
AlcCaM/ANVC	ND	ND	276
AlcCaM/VNAC	ND	ND	489

^a CaM concentration determined by RIA.

^b CaM concentration determined by quantitative Western analysis.

^c NA, not applicable.

^d ND, not determined due to antibody cross-reactivity.

VCaM and AnCaM is that VCaM exhibits an eightfold increase in K_{CaM} for CMKA. Additionally, the V_{max} of VCaM is reduced to about one-half for CMKA and one-third for CMKB. Therefore, the qualitative results of the CaM overlay are not predictive of the ability of VCaM to activate CMKA and CMKB.

Activation of *A. nidulans* CaMKs correlates with the ability of heterologous CaMs to support fungal growth. To evaluate the abilities of heterologous CaMs or the AnCaM 1234 mutant to complement the loss of endogenous CaM in *A. nidulans*, we ectopically expressed different CaM proteins under the control of the constitutive *gpdA* promoter in the background of the AlcCaM strain. In the AlcCaM strain, the expression of the essential CaM gene is regulated by carbon source. In glucose, expression is repressed and the fungus fails to grow. In glycerol, expression is low and the fungus is able to maintain growth (23). Multiple strains expressing the heterologous CaMs were generated, and the expression levels were examined by quantitative Western analysis or radioimmunoassay (Table 3). In glucose, the VCaM and ScCaM expression is similar to the levels of endogenous CaM in the parental GR5 strain, and all these values are similar to previously reported CaM concentrations (40, 23), while the Ca²⁺ binding mutant (1234) is expressed at a concentration about seven times higher than that of endogenous CaM.

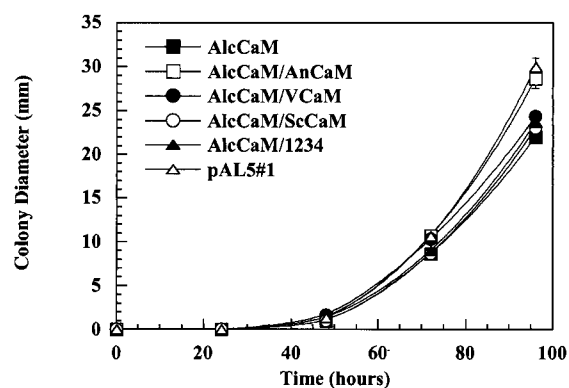
Next, we wanted to determine how well the ability of CaM to activate CMKA and CMKB correlated with its ability to support growth in the absence of endogenous CaM. As seen in Fig. 4A, all strains grew at a rate similar to that of the control when germinated on glycerol medium (allowing wild-type CaM

TABLE 3. Activation of *A. nidulans* CaM kinases

CaM	CMKA		CMKB	
	K_{CaM} (nM)	V_{max} (nmol/min/mg)	K_{CaM} (nM)	V_{max} (nmol/min/mg)
AnCaM	1.9 ± 0.9	77.0 ± 1.6	88.6 ± 24.7	14.1 ± 2.2
VCaM	15.9 ± 7.1	33.2 ± 3.2	133.1 ± 34.7	4.3 ± 0.4
ScCaM	37.8 ± 8.5	9.4 ± 2.8	ND	ND
1234	ND ^a	ND	ND	ND
ANVC	2.1 ± 0.4	50.5 ± 9.5	94.3 ± 18.1	11.6 ± 3.2
VNAC	7.7 ± 0.6	56.1 ± 3.8	102.9 ± 16.3	10.3 ± 2.6

^a ND, no detectable activity

A.



B.

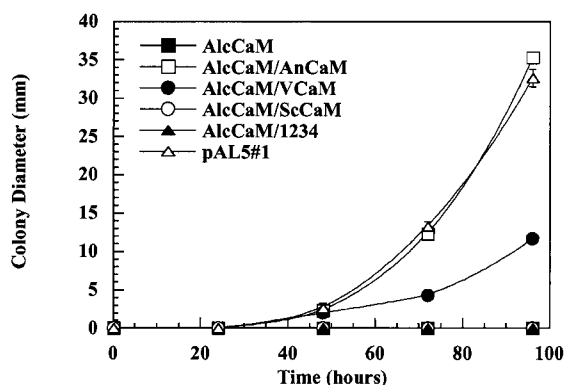
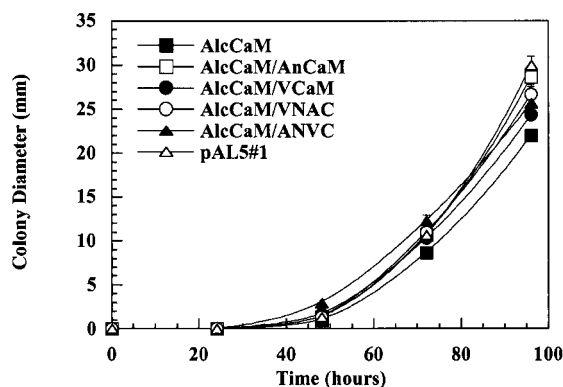


FIG. 4. Complementation of *A. nidulans* by heterologous CaMs. AlcCaM strains expressing the ectopically expressed AnCaM (open squares), VCaM (closed circles), ScCaM (open circles), and 1234 (closed triangles) were inoculated onto either MMG (A) or MMD (B) agar plates, and their colony diameters were measured over a period of 5 days. A nutritionally complemented pAL5#1 strain (open triangles) was used to represent wild-type *A. nidulans* (positive control), and the nutritionally complemented AlcCaM strain (closed squares) was used as a negative control.

expression). When wild-type CaM expression was repressed by the presence of glucose, as demonstrated in Fig. 4B, the AnCaM/AlcCaM strain and the control strain grew at comparable rates, while the nutritionally complemented AlcCaM strain without any heterologous CaM failed to grow. Importantly, neither AlcCaM/ScCaM nor AlcCaM/1234 grows in the absence of *A. nidulans* CaM. Thus, ScCaM and 1234 are essentially inactive in biochemical assays *in vitro* (CaM overlay and CaMK activation), and those results correlate with their inability to support growth *in vivo*.

We also evaluated whether VCaM could functionally complement the absence of the endogenous *A. nidulans* CaM. In glucose, AlcCaM/VCaM grows at a rate approximately one-

A.



B.

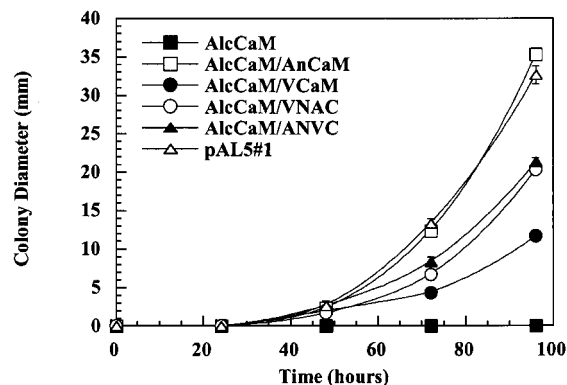


FIG. 5. Complementation of *A. nidulans* by *A. nidulans*/vertebrate chimeric CaMs. The AlcCaM strains expressing AnCaM (open squares), VCaM (closed circles), VNAC (open circles) and ANVC (closed triangles) were grown on either MMG (A) or MMD (B) agar plates, and growth was quantified by measuring colony diameter over a period of 5 days. The nutritionally complemented pAL5#1 strain (open triangles) was used to represent the wild-type *A. nidulans* (positive control), and the nutritionally complemented AlcCaM strain (closed squares) was used as a negative control.

third that of both AlcCaM/AnCaM and the nutritionally complemented control. Thus, VCaM partially performs the essential functions of wild-type CaM *in vivo*, a result that is consistent with its ability to partially activate the *A. nidulans* CaMKs *in vitro*.

The N terminus of CaM is critical for proper activation of CMKA and CMKB. Since VCaM cannot fully complement *A. nidulans* CaM despite sharing 84% amino acid identity, we questioned whether the amino acid differences responsible could be localized to the N- or C-terminal lobe of the protein. The C terminus is more divergent between these species, so we predicted that it would be most important in determining functional differences *in vitro* and *in vivo*. We created two chimeric CaM cDNAs in which the N- and C-terminal globular domains of the *A. nidulans* and vertebrate CaM are interchanged. The junction of the proteins (Fig. 1) is following Asn 61, so the chimeras generated are the *Aspergillus* N terminus-vertebrate C terminus (ANVC) or the *Aspergillus* C terminus-vertebrate N terminus (VNAC) proteins. First, we tested the activation of CMKA and CMKB by ANVC and VNAC (Table 3). Notably, the K_{CaM} for CMKA using ANVC is similar to that obtained with AnCaM rather than to that obtained with VCaM. In contrast, the K_{CaM} using VNAC falls between the values obtained with AnCaM and VCaM. Both chimeric CaMs demonstrate intermediate effects on V_{max} for CMKA. Interestingly, the chimeras display only subtle differences in ability to activate CMKB and are most similar to AnCaM. These biochemical assays support two conclusions: first, the activation of CMKA is more sensitive to differences in the CaM sequence than that of CMKB, and second, amino acid variations in the N terminus, not the C terminus, of VCaM are the most important in determining the K_{CaM} for activation of CMKA.

Both the N and C termini of AnCaM are required for normal growth. If the activation parameters of CMKA *in vitro* correlate with the ability to support growth *in vivo*, then ANVC would be better able to support the growth of the fungus than VNAC in the absence of endogenous CaM. To test this, the cDNAs were expressed in the AlcCaM strain, and their ability

to support growth was compared to that of AnCaM and VCaM. When germinated in glycerol, all of the strains grew at approximately the same rate (Fig. 5A). However, when germinated in glucose, which represses expression of the endogenous CaM, the strains containing the chimeric proteins at two to four times the level of endogenous CaM (Table 2) grew to an intermediate diameter two-thirds that of the wild type but significantly greater than that of AlcCaM/VCaM (Fig. 5B). Thus, even though ANVC and VNAC demonstrate differences in biochemical properties *in vitro*, these two chimeric proteins behave similarly *in vivo* and neither can completely complement the absence of endogenous CaM.

DISCUSSION

In this study, we investigated the ability of the Ca^{2+} binding mutant of AnCaM (1234) and heterologous CaMs from yeast and vertebrate systems to complement the functions of AnCaM biochemically and genetically. As predicted based on the identification of multiple and essential Ca^{2+} /CaM-dependent enzymes in *A. nidulans*, 1234 is unable to activate CMKA or CMKB and cannot support growth of the fungus. Therefore, as with *S. pombe* (30) and in contrast to *S. cerevisiae* (13), CaM of *A. nidulans* requires high-affinity Ca^{2+} binding to support growth. Similar to 1234, ScCaM was unable to activate CMKA and CMKB or functionally complement the absence of the endogenous protein. This inability of ScCaM to complement AnCaM may be due to the significant amino acid alterations that occur throughout the two proteins, the inability of the fourth EF hand to bind Ca^{2+} , or a combination of both types of differences.

Despite sharing 84% amino acid identity with AnCaM, VCaM is unable to fully substitute for AnCaM *in vitro* or *in vivo*. Although the CaM overlays showed that AnCaM and VCaM interact with similar spectra of proteins present in *A. nidulans* crude extracts, VCaM cannot maximally activate either of *A. nidulans*' essential Ca^{2+} /CaM-dependent protein kinases, CMKA and CMKB. For both enzymes, the V_{max} is

reduced relative to AnCaM, but the most striking defect is an eightfold increase in K_{CaM} for CMKA. In correlation with these biochemical results, VCaM only partially complements the loss of AnCaM in vivo. This result is in stark contrast to results of studies with both budding and fission yeast, where VCaM and the endogenous CaM are functionally interchangeable in vivo (5, 30, 34).

Between AnCaM and VCaM, there are only 23 amino acid differences, 13 of which are conservative. Examination of the contacts between vertebrate Ca^{2+}/CaM - and CaM binding peptides based on available three-dimensional X-ray crystal and nuclear magnetic resonance structures reveals that only five of the variant residues interact with CaM binding peptides (10, 19, 25, 26, 35), whereas the other 19 residues lie outside of the CaM/CaM binding peptide interfaces. Although the residues involved in peptide binding may be implicated in the inability of VCaM to complement AnCaM, results of prior biochemical and genetic studies argue that the solvent-exposed residues of CaM are also critical for enzyme activation. Biochemically, both AnCaM/VCaM chimeras are able to rescue the ability of VCaM to activate CMKB. However, the results with CMKA are markedly different. For CMKA the amino acid variation in the N-terminal globular domain is largely responsible for the inability of VCaM to fully activate the kinase. The K_{CaM} for ANVC is similar to that of AnCaM, while the K_{CaM} for VNAC is much greater than that of AnCaM. Within the N-terminal domain of AnCaM and VCaM there are only nine amino acid differences. Intriguingly, amino acids 9 to 12, just prior to the first EF hand, differ in three out of four residues. For AnCaM these residues are VSEY, but for VCaM (as well as for ScCaM and the CaMs of several other lower eukaryotes) these residues are IAEF. Both Ala10 and Phe12 interact with CaM binding peptides in crystallographic studies, and mutation of Phe12 to Ala reduces the ability of ScCaM to activate both *S. cerevisiae* calcineurin and Cmk1p (16). Based on these results, it seems reasonable to suggest that this four-amino-acid cluster may be functionally important. Another difference of potential significance lies at the fourth residue of the second EF hand, Asn in AnCaM and Gly in most other CaMs. Genetic studies of *Paramecium* identified a mutation at this position (Gly59 to Ser) that abrogates the function of Ca^{2+} -dependent Na^+ channels in vivo (21). Interestingly, the Gly-to-Asn variation is also found in the fourth position of the third EF hand in AnCaM. Although these amino acid differences can be speculated to result in biochemical and/or biological consequences, the actual residue or residues responsible for the functional differences cannot be conclusively identified based solely on sequence or structural analysis, since variation in residues within the hydrophobic pockets, on the outer surface of the CaM molecule, or in internally located residues that contribute to stabilizing the structure of the molecule are all potential culprits.

Amino acid substitutions in both globular domains of CaM contribute to the inability of VCaM to fully support growth. Surprisingly, both ANVC and VNAC support *A. nidulans* growth to roughly the same degree, better than VCaM but not as well as AnCaM, even though the biochemical deficits of VCaM can be largely attributed to the N-terminal globular domain. Although the activation of CMKA and CMKB correlates with the ability of ScCaM, VCaM, and 1234 to support

growth in vivo, the analysis of the AnCaM/VCaM chimeras indicates that the in vivo phenotype cannot be simply attributed to the efficacy of these proteins to activate CaMKs. Indeed, several studies suggest that reductions in CaMK activity may not severely impact the in vivo functions of the enzyme. Previous work in our laboratory has demonstrated that for *A. nidulans*, a discernible growth phenotype is observed only when CMKA protein expression (and presumably activity) is repressed to undetectable levels (7). Similarly, in *S. cerevisiae*, biochemical and in vivo analyses of CaM Phe-to-Ala mutants demonstrate that mutations that subtly attenuate calcineurin and CaMK activation in vitro do not appreciably alter the ability of yeast to survive an α -factor-induced growth arrest (16).

Perhaps it should come as no surprise that while the in vitro activation of CMKA and CMKB may correlate with the ability of the heterologous proteins to support growth, the in vivo target(s) involved in causing the phenotypes may be considerably more complex. For example, two essential CaM binding proteins in addition to CMKA and CMKB have been identified in *A. nidulans*. The Ca^{2+}/CaM -dependent phosphatase, calcineurin, and MyoA, a class I myosin, are each required for fungal growth (39, 24). Additionally, a recently identified homologue of *S. cerevisiae* Spc110p and *S. pombe* Pcp1p would be predicted to perform an essential mitotic role in *A. nidulans* based on the null phenotype in yeast (10a). Therefore, alterations in CaM binding or activation of at least five CaM binding proteins may be contributing to the inability of the heterologous proteins to functionally replace the endogenous *A. nidulans* CaM. It will be an interesting challenge to dissect the multiple, essential functions of Ca^{2+}/CaM -dependent and -independent pathways in the growth of this fungus.

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