Cloning of a Ca\textsuperscript{2+}/calmodulin-dependent protein kinase gene from the filamentous fungus *Arthrobotrys dactyloides*

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Abstract

A Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMK) gene was cloned and characterized from *Arthrobotrys dactyloides*, a nematode-trapping fungus. The resulting 373-amino-acid protein, FCaMK, has significant homology to mammalian CaMKs. FCaMK contains a serine/threonine kinase domain followed by a calmodulin-binding domain. The activation loop in FCaMK (amino acids 184–199) contains a phosphorylation site at threonine-188, which could be the target of a kinase activator. Truncated FCaMK mutants revealed that amino acids 296–324 are essential for calmodulin binding. An oligopeptide designed from residues 297–324 formed a stable peptide–calmodulin complex of 1:1 stoichiometry. Southern blot analysis detected a single copy of the *fcamk* gene, suggesting that FCaMK plays an important role in Ca\textsuperscript{2+}/calmodulin signaling in *A. dactyloides*. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Signal-induced changes in the free-Ca\textsuperscript{2+} concentration in the cytoplasm has been portrayed as a second messenger that activates various cellular processes in plants and animals [5,7,27]. Ca\textsuperscript{2+}-mediated protein phosphorylation is one of the major mechanisms by which eukaryotic cells transduce extracellular signals into intracellular responses. Calmodulin-activated protein phosphatases and protein kinases are involved in amplifying and diversifying the action of Ca\textsuperscript{2+}-mediated signals [12,25]. Although little is known about the mechanism of Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CaMKs) in fungi, many of the signal-induced responses have been well documented in controlling spore germination and differentiation [14], cell division [13,23], and protein sorting in the secretory pathway [2].

Nematode-trapping fungi produce specialized structures to trap nematodes and later develop infectious hyphae that grow and occupy the whole body of their prey and consume it in a few days. The most elaborate of these traps are the constricting ring types discovered by Drechsler in 1937. Constricting rings consist of three curved cells on a two-celled stalk. When a nematode passes through the ring, the three cells that make up the ring can triple in volume in 0.1 s to close the opening and catch the nematode [8,19]. The rapid-inflation process is mediated by changes of cytosolic Ca\textsuperscript{2+} levels and by the activities of calmodulin-regulated components in the cells of the *Arthrobotrys dactyloides* constricting rings [6]. In this study, we describe the cloning and identification of the first CaMK (FCaMK) gene from predacious fungi.

2. Materials and methods

2.1. Fungal culture conditions

Spores of *A. dactyloides* were transferred to petri dishes and incubated for 14 days in 1.7% liquid malt-extract broth (Merck) at 28°C in the dark.
Fig. 1. Alignment of predicted amino acid sequences from the \textit{A. dactyloides} Ca\(^{2+}\)/calmodulin-dependent kinase (FCaMK) and other CaMKs. The sequences are as follows: CgCMK from \textit{C. gloeosporioides} [14], cmkB from \textit{E. nidula} [13], SSL2 from \textit{Y. lipolytica} [2], cmk1 from \textit{S. pombe} [23], CAMKI from \textit{H. sapiens} [3], CamK1b from \textit{M. musculus} (GenBank accession number NM_012040), and CaM kinase I from \textit{R. norvegicus} [22]. The roman numbers I-XI above the FCaMK sequence indicate sites that contain the 11 conserved protein kinase catalytic subdomains for CaMKs; the calmodulin-binding domain is indicated by asterisks; and the putative activation loop motif is indicated by underlined. The FCaMK cDNA sequence has been assigned GenBank accession number AF214654.
2.2. Cloning of a cDNA segment encoding CaMK by reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA from the hyphal cultures was isolated using the Ultraspex RNA reagent system (Biotecx). The resulting total RNA was used as a template to amplify the kinase with the following degenerate primers: sense primer, 5'-TCTAGA(C/T)TNGGN(A/T)CGGGN-(A/T)GCTAT(A/T)-CC-3' (based on the amino acid sequence of conserved domain I of serine/threonine protein kinases, LGSGAFS); and antisense primer, 5'-AAGCCTT-(A/G)TANCC(A/G)CANA(A/G)(A/T/G)ATGT-A-3' (based on the amino acid sequence of conserved domain IX, YILLCGY).

The amplification reaction mixture (Promega) contained 1 × AMV/Tf1 reaction buffer, all four dNTPs (each at 200 μM), 1 mM of each primer, 0.5 mM MgSO4, 2 μg of total RNA, 5 U of AMV reverse transcriptase, and 5 U of Tf1 DNA polymerase in a 50-μl total reaction volume. The first cycle consisted of reverse transcription of the RNA template: 45 min at 48°C followed by 2 min at 94°C to terminate the synthesis reaction. The remaining 40 cycles consisted of the following steps: 30 s at 94°C, 1 min at 60°C, and 2 min at 68°C. PCR products were cloned into the vector pBluescript II KS(+) (Stratagene). Clones were sequenced with an automated sequencer (Applied Biosystems). An analysis of nucleotide homology was carried out using the Genetics Computer Group program. The PCR product with the greatest homology to mammalian CaMKs was used as a probe to screen a cDNA library.

2.3. Isolation of FCaMK cDNA clone

An oligo(dT)-primed cDNA library in Uni-ZAP XR (Stratagene) generated from hyphal culture RNA was constructed according to the manufacturer’s instructions. About 5 × 10⁵ plaques were screened using a digoxigenin-labeled (Boehringer Mannheim) 582-bp PCR product (isolated in the previous step) as the probe. From a positive clone was excised to yield pBluescript II KS(+) (Stratagene). Clones were sequenced with an automated sequencer (Applied Biosystems). An analysis of nucleotide homology was carried out using the PCR method. The mutant construct 1–295 was produced by using the sense primer 5'-GGAATTCAATGGCATTCAACCAGCACC-3' with a start codon (the EcoRI site is underlined) and antisense primer 5'-GCGGATCTCATCTCTGCTGTTCTCCG-3' with a stop codon (the XhoI site is underlined); and the mutant construct 1–324 was created by using the sense primer 5'-GGAATTCATTCAACCAGCACC-3' and the antisense primer 5'-GCGGATCTCATCTCTGCTGTTCTCCG-3' and the antisense primer 5'-GCGGATCTCATCTCTGCTGTTCTCCG-3' and the antisense primer 5'-GCGGATCTCATCTCTGCTGTTCTCCG-3'. The PCR conditions were as follows: an initial denaturation step at 95°C for 4 min; 35 cycles of 1 min at 95°C, 2 min at 50°C and 2 min at 72°C; and a last elongation step at 72°C for 10 min. These constructs were then cloned with EcoRI–XhoI into the pGEX-6p-1 expression vector (Pharmacia). Wild-type FCaMK and the mutant proteins were expressed in Escherichia coli BL21(DE3) and purified using glutathione Sepharose 4B (Pharmacia) according to the manufacturer’s instructions.

2.6. Calmodulin-binding assays

Binding assays were performed using calmodulin-immobilized agarose (Calbiochem), calmodulin-binding buffer (40 mM Tris–HCl, pH 7.5; 1 mM CaCl2, 1 mM dithiothreitol, 150 mM NaCl, 0.05% Tween 20 and 10% ethylene glycol) and 4 μg recombinant FCaMK protein (either wild-type or mutant). Assay variables included the addition of 1 mM CaCl2, 1 mM EGTA or 100 μM triluoperazone. The assay mixture (with a final volume of 350 μl) was incubated at room temperature for 30 min with gentle shaking. After centrifugation at 800 × g for 5 min, the matrix pellet was washed with 1 ml calmodulin wash buffer (40 mM Tris–HCl, pH 7.5; 1 mM dithiothreitol, 150 mM NaCl, 0.05% Tween 20 and 10% ethylene glycol). The matrix was then centrifuged at 800 × g for 5 min and washed three more times as above. To elute the bound proteins, 50 μl 10 mM EDTA (pH 7.5) was added to the matrix and incubated for 10 min. The matrix was centrifuged at 800 × g for 5 min and the supernatant was added to an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer and heated at 95°C for 3 min. The resulting proteins were analyzed by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining.

2.7. Peptide–calmodulin-binding assay

The binding assay was preformed as described [1].
Briefly, samples containing 25 μM calmodulin (Calbiochem), different amounts of a high performance liquid chromatography-purified synthetic peptide (ADLLPT-VRKNFARRTLHAAIDTIRAIN, residues 297–324 of FCaMK) in 100 mM Tris–HCl (pH 7.5), and either 0.1 mM CaCl₂ or 2 mM EGTA in a total volume of 30 μl were incubated for 30 min at room temperature. A one-half volume of 50% (v/v) glycerol with 0.1% bromophenol blue was added, and samples were then loaded onto non-denaturing slab gels containing 12% acrylamide in 375 mM Tris–HCl (pH 8.8) and either 0.1 mM CaCl₂ or 2 mM EGTA and electrophoresed at a constant current of 30 mA in running buffer (25 mM Tris–HCl, pH 8.3; 250 mM glycine; and 0.1 CaCl₂ or 2 mM EGTA). The gels were stained with Coomassie brilliant blue.

3. Results and discussion

We used an RT-PCR approach to isolate a DNA segment encoding FCaMK. Degenerate oligonucleotides coding for the conserved amino acid sequences LGSGAFS from domain I and YILLCGY from domain IX of mammalian CaMKs [20,22] were used for RT-PCR with RNA extracted from a hyphal culture of A. dactyloides. This approach yielded six different PCR products, one of which was 582 bp in length, and all six PCR products were cloned and sequenced (data not shown). The 582-bp clone showed a high level of homology with regions from domain I through domain IX of mammalian CaMKs. This cDNA segment was used to screen a cDNA library prepared from a hyphal culture. The resulting putative CaMK-encoding clone, designated FCaMK, was 1820 bp long and contained a single open reading frame encoding a putative protein of 373 amino acids with an estimated molecular mass of 41 kDa.

The deduced amino acid sequence of FCaMK is shown in Fig. 1. Alignment of FCaMK so as to optimize homology to the predicted amino acid sequences of CaMKs from various species gave the following identities: 82% with CgCMK from Colletotrichum gloeosporioides [14], 76% with cmkB from Emericella nidula [13], 60% with cmk1 from Schizosaccharomyces pombe [23], 43% with SSL2 from Yarrowia lipolytica [2], 45% with CAMKI from Homo sapiens [3], 45% with CamK1b from Mus musculus (GenBank accession number NM_012040), and 45% with CaM kinase I from Rattus norvegicus [22,29] (Fig. 1). FCaMK contains the 11 conserved serine/threo-
nine protein kinase domains as well as the calmodulin-binding domain found in other CaMKs [4,10,21]. The full-length fragment of FCaMK was prepared to probe genomic DNA from A. dactyloides. Southern blot analysis (Fig. 2) revealed one gene that hybridized to the full-length FCaMK probe (i.e. Fcamk is a single-copy gene).

A comparison of residues 184–199 of FCaMK with homologous regions from other CaMKI members (Fig. 3) showed the following similarities: CaMKI, 75%; cAMP kinase, 62.5%; and insulin-dependent protein kinase 1, 50%. Within the activation loop, FCaMK also contains a phosphorylation site (Thr-188) in a position similar to that in CaMKI (Thr-177) [22], cAMP kinase (Thr-197) [15], and insulin-dependent protein kinase 1 (Thr-185) [26]. Phosphorylation of threonyl or tyrosyl residues in activation loops purportedly leads to structural stabilization/ordering of the loops, thus promoting increased catalytic activity of these kinases [9,11,16,17]. This is based on the reports that CaMKI is activated by CaMK kinase [18,28]. Further observations supporting this proposal include the activation of insulin-dependent protein kinase 1 by phosphorylation of Thr-185 by mitogen-activated protein kinase [26]. These data suggest that CaMK kinase(s) and phosphatase(s) might be involved in the phosphorylation and dephosphorylation of FCaMK’s activation loop, and that its kinase activity may be regulated by a kinase cascade pathway.

To identify the calmodulin-binding region of FCaMK, truncated mutant constructs were prepared (Fig. 4a). The FCaMK mutant 1–324 lacks the carboxyl-terminal domain, which is of unknown function. Another FCaMK mutant, 1–295, is further truncated but it retains all 11 domains conserved in serine/threonine protein kinases. Wild-type FCaMK (1–373) and truncated mutants 1–295 and 1–324 were expressed in E. coli strain BL21 (DE3) and subsequently used to study calmodulin binding. Similar calmodulin binding was observed for wild-type FCaMK and the mutant 1–324, but no binding was evident for mutant 1–295 (Fig. 4b). Adding either EGTA (a Ca2⁺-specific chelator) or trifluoperazine (a broad-spectrum calmodulin antagonist) led to significant inhibition of calmodulin binding among all the recombinant proteins (Fig. 4b). These observations indicate that residues 296–324 of FCaMK are able to recognize calmodulin in the presence of Ca2⁺ ions.

Calmodulin binding to a 28-residue oligopeptide (ADLLPTVRKNARRTLHAAIDTIRAIN) derived from residues 297–324 of FCaMK was studied by gel mobility shift assay using non-denaturing PAGE. The results (Fig. 5) showed that the peptide is capable of forming a stable complex with calmodulin in the presence of Ca2⁺ but not in the absence of Ca2⁺ (i.e. in the presence of 2 mM EGTA). Several molar ratios of peptide to calmodulin were used. In the absence of peptide, a single band representing free calmodulin was observed. As peptide was added, another band of lower mobility appeared that represented the peptide–calmodulin complex. When equivalent amounts of peptide and calmodulin were present, nearly all the calmodulin was found in the peptide–calmodulin band, and at a peptide/calmodulin ratio of 1.5:1, no free calmodulin was detected. At ratios up to 2.5:1, no change in intensity of the peptide–calmodulin band was detected, nor were any new bands observed, suggesting that multivalent complexes do not form. These observations indicate that the peptide binds Ca2⁺/calmodulin with a 1:1 stoichiometry.

Fig. 4. Deletion studies of the FCaMK calmodulin-binding domain. (A) Schematic diagram of wild-type FCaMK and truncation mutants. (B) Calmodulin-agarose-binding assays with wild-type FCaMK and truncation mutants. The mutants 1–295 lack the calmodulin-binding domain. E. coli-expressed wild-type, mutant 1–295, and mutant 1–324 FCaMK were present in lane 1. Reagents were added to the other lanes as follows: lane 2, 1 mM CaCl₂; lane 3, 1 mM EGTA; lane 4, 100 µM trifluoperazine.

Fig. 5. A synthetic oligopeptide of residues 297–324 of FCaMK forms a stable complex with calmodulin. Complex formation between calmodulin and the peptide was assayed in the presence of 0.1 mM CaCl₂ (top) or in its absence (i.e. in the presence of 2 mM EGTA, bottom). Increasing amounts of the peptide (peptide/calmodulin molar ratios are indicated) were incubated with 250 µM bovine calmodulin, and then samples were separated by non-denaturing PAGE. Arrows indicate the positions of free calmodulin and the calmodulin-peptide complex.
References


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