Cloning and Nucleotide Sequence of the Calmodulin-Encoding Gene (cmdA) from Aspergillus oryzae

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Received January 23, 1995

A cDNA and genomic gene encoding calmodulin were isolated from Aspergillus oryzae using a part of the calmodulin gene from A. nidulans as a hybridization probe. The gene was in a 3.4-kb SpfI fragment and Southern-blot analysis of genomic DNA suggested the existence of a single copy of the calmodulin gene in A. oryzae. The nucleotide sequence analysis showed that the gene consists of five introns and six exons. Although the nucleotide sequence homology with that of A. nidulans was not so high (68%), the deduced amino acid sequence was 100% and 84% identical with calmodulin of A. nidulans and chicken, respectively. The cDNA encoding A. oryzae calmodulin was expressed under the control of the GALI promoter in the calmodulin null mutant (cmdl) of yeast, Saccharomyces cerevisiae, and could function as a calmodulin gene.

Calmodulin, one of the major intracellular Ca2+ receptors, is a small acidic protein found in all the eukaryotes examined.1 The Ca2+-calmodulin complex has been shown to activate many enzymes,2,3 such as phosphodiesterases, Ca2+–ATPase, protein kinases, and adenylate cyclase. Calmodulin also has important regulatory functions in the control of cell growth and cell cycle progression. Calmodulin has been shown to be highly conserved both functionally and structurally. The genes coding for calmodulin have been cloned and characterized in many organisms: eel,4 frog,5 Dictyostelium discoideum,6 chicken,7 Trypanosoma,8 Saccharomyces cerevisiae,9 rat,10 Schizosaccharomyces pombe,11 Chlamydomonas reinhardtii,12 Achlya klebsiana,13 Aspergillus nidulans,14 and Candida albicans.15

Aspergillus oryzae is a very important strain in traditional Japanese food industries, like those making sake, soy sauce, and miso. Recently, members of the genus Aspergillus have received considerable interest as the hosts for heterologous gene expression and foreign protein production. Several genes were isolated from A. oryzae: x-amylose (amyB),16 glucoamylase (glaA),17 ribonuclease T2 (mmT),18 acid protease (pepA),19 acid protease (aaps),20 and alkaline protease.21,22 and neutral protease.23 To obtain many molecular biological understandings of A. oryzae, it is necessary to clone more genes and to study the molecular structure and function of those genes.

We report here the isolation and characterization of a calmodulin-encoding gene from A. oryzae. We have initiated studies to obtain a better understanding of gene expression and to examine the role of calmodulin involved in morphogenesis.

Materials and Methods

Strains, plasmids, and media. A. oryzae RIB40 was used as a DNA and messenger RNA donor. E. coli P2392,24 IM109,25 plasmids pUC18,26 pUC19,26 and the phage vector λEMBL327 were used for DNA manipulation. Saccharomyces cerevisiae YOC101 (a ura3 lys2 ade2 trpl his3 leu2 cmdl-1::TRPl, harboring plasmid pBR1612 (CEN6 URA3 CMD1) was described before28 and used as a host for expression of A. oryzae calmodulin cDNA. A yeast expression plasmid, YcpLGNN87 (Y. Ohya, unpublished) contained a GALI promoter and a LEU2 marker on a CEN plasmid, was used. Dextrin-peptone medium contains 2% dextrin, 1% polypeptide, and 1% yeast extract was used for cultivation of A. oryzae. For cultivation of S. cerevisiae,YPD medium (1% yeast extract, 2% polypeptide, and 2% glucose), and YM medium (0.67% yeast nitrogen base without amino acid (YNB) (Difco, U.S.A.), and 2% glucose) were used. For A. oryzae calmodulin complementation experiments, YM-trp-ura-leu plates (YNB, glucose, lysine, adenine, histidine, and agar), YM-trp-leu + 5-FOA plates (YNB, galactose, lysine, adenine, histidine, uracil, 5-fluoroorotic acid, and agar), YM-Glc-ura plates (YNB, glucose, lysine, adenine, histidine, leucine, and agar), YM-Glc-leu plates (YNB, glucose, lysine, adenine, histidine, leucine, and agar), and YM-Gal-ura plates (YNB, galactose, lysine, histidine, uracil, and agar) were used. The concentrations of added media used are as follows; uracil (20 μg/ml), lysine-HCl (30 μg/ml), adenine (20 μg/ml), histidine-HCl (20 μg/ml), leucine (30 μg/ml), 5-fluoroorotic acid (1 mg/ml) (Sigma, U.S.A.), glucose (2%), galactose (2%), and agar (2%).

Plaque hybridization. DNA probe was radiolabeled with [α-32P]dCTP (Amerham, UK). Plaques were transferred onto a Hybond-N nylon membrane (Amerham, UK) and then fixed by UV-irradiation for 5 min. Hybridization was done at 55°C in a solution containing 6× SSC, 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml of salmon sperm DNA. Following hybridization overnight, the membranes were washed twice for 30 min in 2× SSC/0.1% SDS at 55°C.

Polymerase chain reaction (PCR). PCR amplification was done with 100 ng of the template DNA in 100 μl containing 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH4)2SO4, 6.7 mM MgCl2, 10 mM 2-mercaptoethanol, 200 μM each dATP, dCTP, dGTP, and dTTP, 1× each primer, and 3

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teas" were done by the methods of Hanahan\(^{20}\) and Ito et al.,\(^{31}\) respectively.

**Southern-blot analysis.** About 10 µg of the genomic DNA from *A. oryzae* RIB40 was digested with restriction endonuclease and separated by 0.8% agarose gel electrophoresis. Following transfer to a nylon membrane, hybridization and detection were done using an ECL gene detection system (Amersham), in which the probe was labeled by non-radioactive method.

**Preparation of mRNA.** *A. oryzae* RIB40 was grown in 100 ml of dextrin-peptone medium at 30°C for 3 days with shaking, and harvested through a glass filter. Total RNA was isolated by a modification of method of Cathala et al.\(^{12}\) Poly(A)\(^+\) mRNA was purified from total RNA using an Oligotex-dT30 (Daichi Chemicals).

**Transformation experiments.** The transformation of *E. coli* and *S. cerevisiae* was done by the methods of Hanahan\(^{20}\) and Ito et al.,\(^{31}\) respectively.

**DNA sequencing.** Deletion series for sequencing were generated from unidirectional insertions of the calmodulin gene using a kilo-deletion kit (Takara Shuzo). The nucleotide sequence was identified by the dideoxy chain termination method of Messing\(^{30}\) using an automated DNA sequencer (Applied Biosystems, Model 370A). Both directional strands were completely analyzed by overlapping at every junction.

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**Fig. 1.** Restriction Endonuclease Map of the DNA Fragment Containing the Calmodulin-encoding Gene (cmdA), and Its Nucleotide Sequence and Deduced Amino Acid Sequence.

(A) The protein coding regions (exons) are indicated by solid boxes. The direction of translation is shown by the arrow. **B:** \(B_g\); \(B_{Hg}\); \(H_{Hg}\); \(H_{rc}\); \(N_{rc}\); \(N_{r}\); \(P_{rc}\); \(P_{r}\); \(Sp_{ry}\); \(St_{r}\); \(St_{l}\); \(Xb_{rc}\); \(Xb_{r}\)

(B) Nucleotides are numbered with reference to the translation start codon (+1). TATA-like and CAAT-like elements in the 5′-noncoding region and conserved 5′ internal and 3′ consensus sequence in introns are underlined. Protein coding sequences are indicated in uppercase letters and all other sequences in lowercase letters. The deduced amino acid sequence is shown below each codon. The sequence data presented here has been submitted to the DDBJ/EMBL/GenBank under the accession number D28340.
Results

Isolation of calmodulin genomic DNA

The 460-bp DNA fragment was amplified by PCR and used as a hybridization probe. Approx. 10,000 plaques of the 2EMBL3 genomic library of A. oryzae RIB40 were screened and 10 positive clones were isolated. Subcloning analysis showed that the 3.4-kb Spel fragment contains the entire calmodulin gene, hereafter called cmdA (Fig. 1A). Southern blots of genomic DNA digested with several restriction endonucleases were probed with the 3.4-kb Spel fragment (Fig. 2). In each digest, a single hybridizing band was detected, suggesting that the cmdA gene exists as a single copy in A. oryzae RIB40.

Isolation of calmodulin cDNA

Calmodulin cDNA was isolated by RNA PCR (see Materials and Methods). The mRNA was prepared from A. oryzae RIB40 and oligonucleotides primers derived from the genomic sequence were synthesized. The upstream primer was derived from the 5'-noncoding region (5'-AA-AAGATCCTTATCCCTCCACATTGT3') and the downstream primer was an antisense primer derived from the 3'-noncoding region (5'-AAGGATCCGGGTATAAAGGGAATAT3'). Both primers contained a BamHI site. The calmodulin cDNA was reverse-transcribed from the mRNA and amplified as a 500-bp BamHI fragment. This fragment was subcloned into pUC118.

Nucleotide sequence of the cmdA gene

Figure 1B shows nucleotide sequences of the genomic clone (2290 bp Spel-BgII fragment) and cDNA of calmodulin. Comparison of cDNA to the genomic sequence identified five putative introns in the gene. Both the 5' splice junction and the 3' splice junction of introns match the consensus splice site sequence identified in filamentous fungi. The 5'-noncoding region contained a TATA-like element (TATTAT)-178 nucleotides from the start codon, and a CAAT-like element (CAAAAT)-378 nucleotides away. No canonical AATAAA polyadenylation signal was present in the 3'-noncoding region of the cmdA gene. Based on this structural analysis, we concluded that the cmdA gene encoded a protein of 149 amino acids.

Comparison of A. oryzae calmodulin with those of other organisms

Alignment of the deduced amino acid sequence of A. oryzae calmodulin showed a high degree of identity with the calmodulins of other organisms (Fig. 3). The A. oryzae calmodulin was found to be 100% identical to A. nidulans calmodulin and 84% identical to chicken calmodulin. However, A. oryzae calmodulin had a low degree of identity with Schiz. pombe (68%) and S. cerevisiae (54%). The putative Ca$^{2+}$ binding domains have a high degree of homology with those of other organisms (Fig. 3).

Fig. 2. Genomic Southern-blot Analysis of A. oryzae DNA. Approximately 10 μg of genomic DNA of A. oryzae RIB 40 was digested with the following restriction enzymes and separated by 0.8% agarose gel electrophoresis, followed by transfer onto a nylon membrane. Southern-blot analysis was done with a 3.4-kb fragment as a probe. Molecular sizes of HindIII-digested lambda phage DNA are shown at the left of the panel. lane 1, EcoRI; lane 2, EcoRV; lane 3, KpnI; lane 4, PstI; lane 5, SalI; lane 6, SmaI; lane 7, Spel; lane 8, XhoI.

Fig. 3. Comparison of the Deduced Amino Acid Sequence of the Calmodulin Gene from A. oryzae with Calmodulin Proteins from Other Organisms. The deduced amino acid sequence of the cmdA gene from A. oryzae was aligned manually with the sequence of Aspergillus nidulans, chicken, Tetrahymena, Achlya klephya, Dictyostelium discoidum, Chlamydomonas reinhardtii, Candida albicans, Schizosaccharomyces pombe, and Saccharomyces cerevisiae. Putative Ca$^{2+}$-binding domains are underlined. Identical residues are represented by a dash. Symbol # indicated two residue gaps introduced into the S. cerevisiae and A. oryzae sequence to give maximal alignment with other calmodulin proteins. The degree of identity is calculated in % of identical amino acid residues with respect to A. oryzae (100%).
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**Fig. 4.** Construction of Calmodulin Expression Plasmid, YCP78T-cCaM in Yeast. The 0.5-kb *NcoI-BamHI* fragment encoding the *A. oryzae* calmodulin cDNA was inserted into the *NcoI-BglII* gap of plasmid YCP378T, yielding YCP78T-cCaM.

**Fig. 5.** Experimental Strategy to Test for *Aspergillus* Calmodulin Complementation of Yeast cmd1 Mutant. *S. cerevisiae* YOC101 was transformed with the plasmid YCP78T-cCaM. To test the ability of the *A. oryzae* gene to complement the *CMD1* disruption, the pRB1612 plasmid was eliminated from the transformants by plating on YM-trp-ura-leu + 5-FOA plate lacking tryptophan and uracil.

**Fig. 6.** Complementation of a Yeast cmd1 Mutation by Expression of *A. oryzae* Calmodulin cDNA. YOC101 (cmd1::TRP1 [pRB1612]) (A, C, E, G) and YKY11 (cmd1::TRP1 [YCP78T-cCaM]) (B, D, F, H) were streaked on a YM-Glc plate without uracil (A, B) or without leucine (C, D), and on a YM-Glc acetate plate without uracil (E, F) or without leucine (G, H). After 4 days of incubation at 30°C, the plates were photographed.

**Complementation of yeast calmodulin mutation (cmd1) by *A. oryzae* calmodulin cDNA**

To construct a plasmid for expression of the *A. oryzae* calmodulin in yeast, the 0.5-kb *NcoI-BamHI* fragment containing the entire calmodulin cDNA was inserted into the *NcoI-BglII* gap of the yeast expression plasmid YCP78T, yielding YCP78T-cCaM, in which the expression of *A. oryzae* calmodulin was regulated by the *GAL1* promoter (Fig. 4). Transcription from this promoter is induced in galactose medium and repressed in glucose medium. YCP78T-cCaM was used to...
transform a yeast strain, YOC101, which harbors a cmd1 deletion (cmd1-Δ1::TRP1) and a wild-type CMD1 gene on a UR3A to keep it alive. The Leu" transformants were then streaked on YM-trp-leu+5-FOA plate. Because only uracil auxotrophs can grow on an FOA plate,35) strains without the plasmid carrying UR3A gene and CMD1 gene only could grow on the FOA plate. Figure 5 shows the protocol for expression of A. oryzae calmodulin in place of S. cerevisiae calmodulin. Figure 6 shows that a yeast strain (YKY11), relying solely on A. oryzae calmodulin expressed under the GAL1 promoter, could grow in a galactose-dependent manner and showed uracil auxotrophy. These results clearly indicated that A. oryzae calmodulin cDNA could function as a calmodulin gene in yeast.

Discussion

We have cloned and characterized the unique calmodulin gene from the filamentous fungus Aspergillus oryzae. Several organisms have multiple calmodulin genes, such as man,37,38) frog, and Trypanosoma, while a unique gene has been identified in Drosophila,39) Dietyostelium, and yeast. In A. oryzae, however genomic Southern analysis suggested that the cmdA exists as a single copy. The nucleotide sequence of the cloned cmdA gene has an open reading frame of 896 bp containing five putative introns, and encodes 149 amino acid residues.

The nucleotide sequence of the putative coding region has a high degree of homology (68%) compared with the corresponding A. nidulans gene. When these five introns were removed from the nucleotide sequence, the homology observed between the calmodulin genes of A. oryzae and A. nidulans was more than 85%. The intron number and inserted positions were same in the calmodulin genes between both species. These observations indicate that both species are closely related phylogenetically.

Analysis of the 5'-flanking sequence of the A. oryzae cmdA gene found no canonical TATA and CAAT elements. Most fungal TATA and CAAT boxes are found 50–100 bp and 100–200 bp from the translation start codon, respective-ly.40) Although a TATA-like sequence (TATTAT) and a CAAT-like sequence (CAAAAT) were found in this gene, both sequences were more than 150 bp and 300 bp distant, respectively. The putative polyadenylation signal (AA-TAAA) was not found in the 3'-flanking sequence. This is not unusual for fungal genes.33) Five introns ranging from 56 to 126 bp were found in the A. oryzae cmdA. Each intron has a 5' GT and 3' AG splice junction characteristic of eukaryotic introns, as well as a sequence related to "RCTRAC," unique to fungal introns. Several calmodulin genes contain introns. In general, intron positioning is found at the 5' end of the gene. It is very interesting that an intron positioned just after the start codon has been conserved in the calmodulin genes of rat, chicken, fly, C. albicans, Schiz. pombe, A. nidulans, and A. oryzae. The A. oryzae calmodulin protein was found to be 84% identical to vertebrate calmodulin. This degree of identity is greater than either Schiz. pombe (68%) or S. cerevisiae (54%), related Ascomycete fungi. Ca2+-binding domains of A. oryzae calmodulin have been shown to be highly conserved structurally. All Ca2+-binding domains appear quite similar to vertebrate calmodulin.

To construct a plasmid for expression of the A. oryzae calmodulin in yeast, the cDNA encoding A. oryzae calmodulin was fused to a galactose-inducible GAL1 promoter. When the expression plasmid was introduced into a calmodulin-gene-disrupted strain of yeast, A. oryzae calmodulin cDNA could function as a calmodulin gene in yeast. Although the amino acid sequence of yeast calmodulin shows only 54% homology with that of A. oryzae calmodulin, this suggests that A. oryzae calmodulin is functionally homologous to yeast calmodulin. In the same system, chicken calmodulin cDNA complemented the cmd1 null mutation of yeast,41) although the amino acid identity between chicken calmodulin and yeast calmodulin is not very high (60%).

We have analyzed the molecular structure of the calmodulin gene from A. oryzae. However, there are no reports on functional analysis of calmodulin in A. oryzae. To identify the calmodulin function in A. oryzae, we are now attempting to construct A. oryzae strains in which calmodulin gene expression is under the control of a regulatable promoter such as the glycoamylase gene (glaA).17)

Acknowledgment. The authors are grateful to Messrs. K. Tsuchiya, M. Ogawa, and T. Minetoki for helpful discussions.

References


