Molecular characterization of a calmodulin-like *Dictyostelium* protein CalB[1]

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Received 22 February 2000; received in revised form 15 April 2000

Edited by Pierre Jolles

Abstract A gene named *calB* was cloned and characterized in *Dictyostelium*. A relationship to calmodulin (CaM) is suggested by sequence identity (50%), similar exon–intron structure and cross-reactivity with anti-CaM sera. The level of *calB* mRNA is developmentally regulated with maxima during aggregation and in spores. CalB null cells grow normally, develop and produce viable spores. We demonstrated the capacity of tagged CalB to bind Ca^{2+} using the $^{45}Ca^{2+}$ overlay assay and showed that its mobility on SDS–PAGE is dependent on Ca^{2+}/EGTA pretreatment.

Key words: Ca^{2+}-binding protein; Calmodulin; EF-hand; *Dictyostelium discoideum*

1. Introduction

A crucial step in many Ca^{2+}-dependent cellular processes is the activation of Ca^{2+}-binding proteins such as calmodulin (CaM). Whereas the role of CaM in cells has received considerable attention, the plethora of other Ca^{2+}-sensing/-binding proteins is only recently being unraveled.

In *Dictyostelium*, changes in cytosolic Ca^{2+} are implicated in regulating chemotaxis, movement and gene expression in both prespore and prestalk cells (for review [1,2]). CaM in *Dictyostelium* seems to be essential for growth [3], it is required for successful germination of spores [4], and it is a prerequisite for the completion of cell and pronuclear fusion during the sexual cycle [5]. The expression of CaM mRNA is constant during development [3].

Several EF-hand-type Ca^{2+}-binding proteins have been identified in *Dictyostelium* in addition to CaM. These novel Ca^{2+}-binding proteins include calfumirin-1 [6], CBP1 [7], CBP2 [8] and CBP3 [9]. All these proteins are small in size (<20 kDa) and contain four EF-hand motifs, similar to CaM. Their Ca^{2+}-binding and physiological properties, however, have not been clearly established. Interestingly, all the CBP proteins show variations in their expression patterns during asexual development [7–9].

The versatility of Ca^{2+} signaling may depend in part on the presence of various Ca^{2+} sensors available in each cell. Here we present the characterization of a novel Ca^{2+} sensor protein, which has the highest similarity to CaM of the known Ca^{2+}-binding proteins of *Dictyostelium*.

2. Materials and methods

2.1. Cultivation and transformation of Dictyostelium discoideum

Dictyostelium cells of the strain AX2 were grown axenically in HL5 medium up to the density of 2×10^6 cells/ml. To obtain cells at various developmental stages, the culture was cooled on ice, centrifuged ($260\times g$ for 10 min) and the cell pellet was washed twice with ice cold 17 mM phosphate buffer (pH 6.0). Cells were plated on non-nutrient agar and allowed to develop at 21°C with overhead light.

The cells were transformed using the calcium phosphate coprecipitation method as described [10]. The transformed cells were selected in 10 μg/ml G418 (Amersham, UK) or 10 μg/ml blasticidin S (ICN, USA), emerging colonies were picked and cultivated independently.

2.2. Cloning and sequencing of the calB gene

We discovered a sequence tag highly homologous to CaM at the *Hin*dIII end of a *Hin*dIII/*Bam*HI fragment of *Dictyostelium* chromosomal DNA carrying the 5′-end of the gene *snwA* (see Fig. 1A; [11]). To isolate a full-length clone of the novel gene, we first used Southern analysis to find suitable restriction sites adjacent to the *Hin*dIII site. Using the PCR-amplified sequence tag as a probe, we identified a 1900 bp *Hin*6I/*Bam*HI fragment. The gel band corresponding to this fragment was excised and ligated into pBluescript SK+ (Stratagene, USA) opened in *Bam*HI and *Cla*I restriction sites. The clone of interest was isolated from this subgenomic library by repeated rounds of subcloning and PCR-aided testing (F. Půta, protocol available upon request). The primers were: sense primer (5′-GCCTC TAGAT ATCAG CTTCA GAATC) and antisense primer (5′-GGCAT GGCAA AAGAA GATAC TAC).

2.3. Northern blot analysis

Total cellular RNA was extracted from both vegetative and developing cells using the acid guanidinium–thiocyanate–phenol–chloroform extraction method as described by Chomczynski [12]. Staged RNA of equal quantity was separated on a 1.2% agarose–6% formaldehyde gel and blotted onto a nylon membrane. Antisense *calB* RNA was prepared as a probe (SP6/T7 Transcription kit, Boehringer Mannheim) and labeled with [^{32}P]UTP. Prehybridization, hybridization and detection were performed as recommended by Boehringer.

2.4. Inactivation of the calB gene in Dictyostelium

The *calB* gene was inactivated by homologous recombination using a blasticidin resistance (BsR) cassette [13]. The selection cassette was subcloned into the *Eco*RV site of the *Hin*6I/*Bam*HI fragment, containing *calB* (see Fig. 1). The fragment was isolated from the vector DNA, Klenow-filled and used for the transformation of *Dictyostelium* AX2 cells (20 μg/transformation). The resistant clones were screened by PCR, the occurrence of homologous recombination was confirmed by Southern analysis of genomic DNA.

2.5. Expression of tagged variants of CalB in Dictyostelium

To express His-tagged and His/myc-tagged variants of CalB in *Dictyostelium*, the full-length open reading frame was PCR-amplified

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[1] The nucleotide sequence reported here has been submitted to the GenBank/EMBL data bank under the accession number AF001981.

Abbreviations: CaM, calmodulin; BsR, blasticidin resistance

PII: S0014-5793(00)01551-9

from a *Dictyostelium* cDNA library (kindly provided by Prof. R.P. Dottin). The primers (5′-GATGGTAC/CATGGCAAAAGAAGATAC) and (5′-GGTC/TCGAGAATTGAATGATTTGCTT) contained *Kpn*I and *Xho*I restriction sites, respectively. The PCR product was cloned into pBluescript SK+, sequenced and recloned into *Dictyostelium* expression vectors pDXA-3H, pDXA-3C and pDXA-HC [14]. The constructs were used for the transformation of *Dictyostelium* AX2 cells.

2.6. Protein analyses

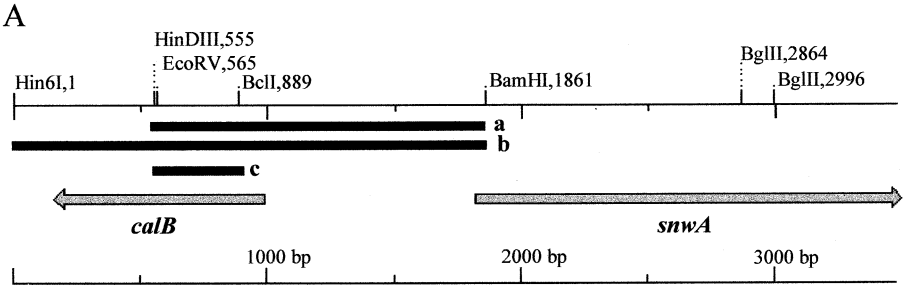
The His-tagged variants of CalB were affinity-purified from vegetative *Dictyostelium* cells using the TALON™ metal affinity resin (Clontech, USA). The cells were grown in axenic shaken cultures to the density of 1×10^7 cells/ml, washed three times in ice cold 17 mM phosphate buffer (pH 6.0) and lysed in lysis buffer (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5% NP-40) by vigorous vortexing. The $20\,000\times g$ supernatant of the lysate was then processed on the cobalt affinity resin. Proteins were separated by high-resolution Tris–Tricine SDS–PAGE [15], transferred onto a PVDF membrane in 25 mM phosphate buffer according to Hulen [16], and fixed in the same buffer containing 0.2% glutaraldehyde. The tagged variants of CalB were detected by anti-myc monoclonal antibody 9E10 (Sigma).

To test Ca^{2+}-binding of CalB, the overlay assay of Maruyama was implemented with some modifications [18]. Briefly, proteins were separated by Tris–Tricine SDS–PAGE and transferred onto a PVDF membrane. The membrane was washed three times in 5 mM imidazole buffer (pH 7.4) and incubated for 20 min in the same buffer containing 5 mM $MgSO_4$, 60 mM KCl and 10 μM $^{45}CaCl_2$ (0.5 μCi/ml). Following the incubation, the membrane was rinsed in 30% ethanol, dried and autoradiographed.

The Ca^{2+}-induced mobility shift of CalB was performed on a 12% Tris–glycine SDS–PAGE gel in the presence of 2 mM $CaCl_2$ or 2 mM EGTA.

3. Results

The full-length genomic DNA and cDNA clones of *calB*



B

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1     TGGGTGAAAAAAAAAAAAAAAAATAAAAAAAATAAAAAAAAAAAAAGGTGTAATATATTTAATCAACATACATTCAG

76    CATATATAATAAAAGCAATGGTAATTTCATCACCCCAATATATATATTTTTTTTATTTTAATTTTTTCAATTTTT
1                    M-----------------------------------------------------

151   TTTTTTTTTTTTTTTTTTTTGATCAACTATATATTAACTCTATTTTTTTTTTTTTAATATTAATTCAAAAAAAAAAA
      ---------------------------------Intron 1.---------------------------------

226   AAAAAAAAAAAAAAAAAAAAAAAATAGGCAAAAGAAGATACTACTCAAGCATTATATAAAGAAGCATTCAATTTAT
2     -------------------------- A  K  E  D  T  T  Q  A  L  Y  K  E  A  F  N  L

301   TTGATAAAGATAAAGATGGTAAAATTACAATTCAAGAATTAGGTATAGTTATGAGATCAGTTGGATCAAACCCAA
18    F  D  K  D  K  D  G  K  I  T  I  Q  E  L  G  I  V  M  R  S  V  G  S  N  P

376   CTCAACAAGAACTTAAAGATATTGCTAAAGAGATTGACGATGGTTCAGGTTTAGTAGATTTTAGTAAATTTTCAT
43    T  Q  Q  E  L  K  D  I  A  K  E  I  D  D  G  S  G  L  V  D  F  S  K  F  S

451   CACTCATGACTAGAAAAATGAAATATTCAGATTCTGAAGCTGATATCAAACAAGCTTTCAAAGTTTTCGATAAAA
68    S  L  M  T  R  K  M  K  Y  S  D  S  E  A  D  I  K  Q  A  F  K  V  F  D  K

526   AAGGTAATGGTTATGCAAATATTCAAGATTTAAAACACACATTAACTTCAATTGGTGAAAAATTAACAAAAGAAG
93    K  G  N  G  Y  A  N  I  Q  D  L  K  H  T  L  T  S  I  G  E  K  L  T  K  E

601   AAGTATGTATTACATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT
118   E-----------------------------------------------------------------------

676   TATTATTATTATTATTATTATTATTATTATTATTATTATTATCATTTTTGAACTCTATTCTAATAATTAAAT
      ---------------------------------Intron 2.---------------------------------

751   TTTCTTTTTTTTTTTTTTTTTTTTTATAACTAAAAATAAAAAAAATAAAAAAAATAAATATAAATTATATTTTAGT
      ------------------------------------------------------------------------

826   TCGATAATATGTTAAAAGACGCAAAAACTGTTGATGGCCAAATTCATGTTGATGAATTTGTTAGAGTTATTAAAT
119   F  D  N  M  L  K  D  A  K  T  V  D  G  Q  I  H  V  D  E  F  V  R  V  I  K

901   CAAGCAAATCATTCAATTAAATAGGAAATGTAATATTAAAAAATAATAATAATAATTATAAAAAAAATAAAAAAA
144   S  S  K  S  F  N  #

976   AGATATAAATATCCATATACACTTATTTATAAGAAAAAAAAAAAAAAAAAAAAAAGGTTTTTATTTATTATTTAC
1051  AAATTCGGCGC
```

Fig. 1. Cloning strategy and sequence analysis of *Dictyostelium calB*. Restriction map of the genomic region of *calB* (A). The bars represent the original *Hin*dIII/*Bam*HI fragment (a), the fragment obtained by genomic cloning (b) and the hybridization probe (c). Genomic DNA and the deduced amino acid sequence of CalB (B). The consensus splicing sites are underlined, the hypothetical TATA box is double-underlined and the *Eco*RV restriction site is shadowed.

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Ca2+ ligating coordinates                x  y  z -y -x  -z
EF-hand consensus                  EΦ--ΦΦ--Φ-----G-Φ---EΦ--ΦΦ--Φ
CalB       MAKED----TTQALYKEAFNLFDKDKDGKITIQELGIVMRSVGS
CaM        MASQESLTEEQIAEFKEAFSLFDKDGDGSITTKELGTVMRSLGQ
           **.::        * :*****.***** **.** :*** ****:*.

CalB                NPTQQELKDIAKEID-DGSGLVDFSKFSSLMTRKMK
CaM                 NPTEAELQDMINEVDADGNGNIDFPEFLTMMARKMQ
                    ***: **:*: :*:* **.* :**.:* ::*:***:

CalB               YSDSEADIKQAFKVFDKKGNGYANIQDLKHTLTSIGE
CaM                DTDTEEEIREAFKVFDKDGNGYISAAELRHVMTSLGE
                    :*:* :*::******.**** .  :*:*.:**:**

CalB                KLTKEEFDNMLKDAK-TVDGQIHVDEFVRVIKSSKSFN
CaM                 KLTNEEVDEMIREADLDGDGQVNYDEFVKMMIVRN---
                    ***:**.*:*:::*.   ***:: ****:::   :
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Fig. 2. Pairwise alignment (CLUSTAL W (1.7)) of amino acid sequences of *Dictyostelium* CaM and CalB. The EF-hand loop regions are shadowed, homologous and identical amino acids are indicated by dots and stars, respectively. The Ca^{2+}-ligating coordinates (*X*, *Y*, *Z*, $-Y$, $-X$, $-Z$) and the consensus sequence for EF-hand motif (E, glutamic acid; Φ, non-polar amino acid; G, glycine) are written on the top of the first EF-hand. The residues that are functionally important in CaM and are conserved in CalB are in bold (tyrosine 99, methionines 36, 72, 76 and 124, and lysine 115; see text for details).

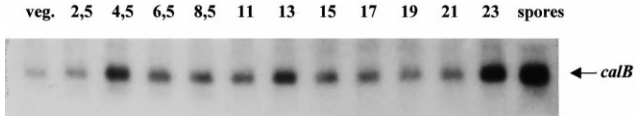

Fig. 3. Developmental regulation of *calB* mRNA expression. The total RNA was extracted at various times of development, fractionated on a 1.2% agarose–6% formaldehyde gel, blotted onto nylon membrane and hybridized with [^{32}P]UTP-labeled antisense *calB* RNA.

were obtained as described in Section 2. The genomic clone spans 1061 bp (Fig. 1B), the consensus TATA box is present 41 bp upstream of ATG, two putative polyadenylation signals can be traced in the 3′-untranslated region. The comparison of genomic and cDNA clones revealed the presence of two A/T-rich introns. The first intron is positioned immediately after the initiation codon, which is a feature characteristic of all CaM genes [19]. The position of the second intron of *calB* corresponds to the position of the last intron of rat myosin light chain (MLC_2), which is again a preferred site in the lineage of the Ca^{2+}-binding protein family [20]. Splicing follows the 5′-GT- - -3′-AG rule. Low stringency Southern analysis revealed the presence of a single copy gene (data not shown).

The *calB* gene encodes a 16.8 kDa protein (calculated p*I* 5.75), which is 51% identical to *Dictyostelium* CaM (65% identity at the cDNA level). The deduced amino acid sequence of CalB contains four EF-hand motifs and fits the consensus for regulatory Ca^{2+}-binding proteins ([20]; Fig. 2). The EF-hand motifs I and III score high in the Pfam database of protein domain family alignments [21]. Other features also support the hypothesis that CalB is a Ca^{2+}-sensing protein. The tyrosine 99 of CaM, which is subject to regulatory phosphorylation, is preserved [22], as well as the lysine 115, the target for methylation [23]. The methionines (36, 72, 76, 124) that form flexible hydrophobic patches that bind helices of CaM substrates [24] are also conserved in CalB.

The Northern analysis of *calB* shows that the gene is developmentally regulated (Fig. 3). The *calB* transcript is very low in vegetative cells, it accumulates to higher levels during aggregation (after 4.5–6.5 h of development) and during slug formation (after 13 h). Interestingly, it is present in its highest level in spores.

To investigate the role of *calB* in *Dictyostelium*, we inactivated the *calB* gene by homologous recombination. The BsR selection cassette was inserted into the second exon, immediately after the first two EF-hand motifs of *calB* (Fig. 1). The disruption did not cause any phenotypic abnormalities, however, we cannot exclude that the *calB*$^-$ cells have a 'less severe' phenotype, which cannot be observed under standard conditions.

We expressed three tagged variants of CalB in *Dictyostelium* (N-terminally His-tagged, N-terminally His-tagged and C-terminally myc-tagged, and C-terminally myc-tagged) to study the in vivo and in vitro properties of the protein and its role in Ca^{2+} signaling. High levels of tagged CalB expression were obtained in all transformants tested. All the CalB overexpressing clones grew normally and no phenotypic alterations were found in development.

The CalB adheres poorly to nitrocellulose, but, similarly to

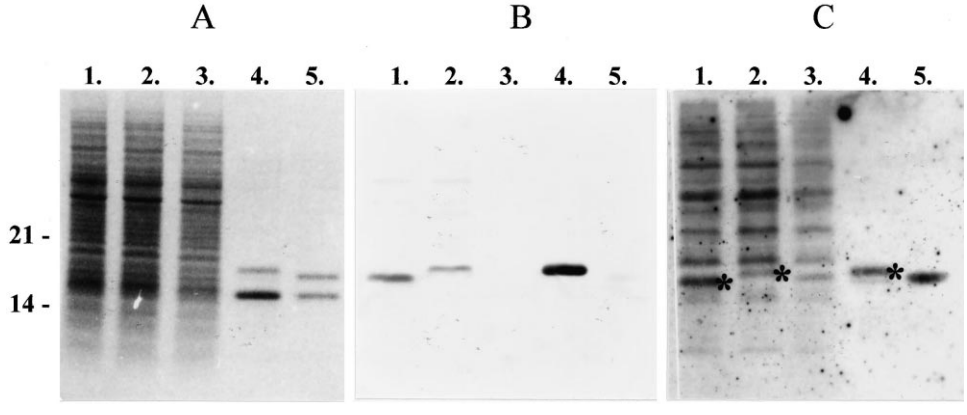

Fig. 4. Immunoblot analysis of CalB expressing clones and $^{45}Ca^{2+}$ overlay assay. Proteins were separated by high-resolution 10% Tricine SDS–PAGE (A, Coomassie blue-stained gel), transferred onto PVDF membrane and the myc-tagged variants of CalB were detected by anti-myc monoclonal antibody (B). The membrane was subsequently overlaid with $^{45}Ca^{2+}$ (C). Lanes 1–3: cell lysate (5×10^5 cells per sample) from cells overexpressing myc-tagged CalB (1), His/myc-tagged CalB (2) and His-tagged CalB (3). Lanes 4 and 5: TALON™ metal affinity resin-purified His/myc-tagged CalB (4) and His-tagged CalB (5). Molecular size markers (kDa) are indicated at the left. The bands marked with the asterisks in C colocalize with the antibody-generated signal in B.

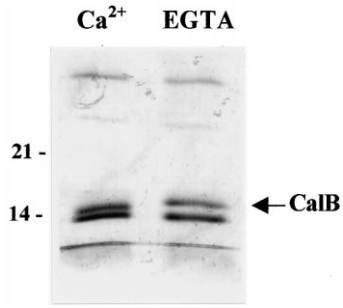

Fig. 5. The effect of Ca^{2+} on the electrophoretic mobility of CalB. His-tagged CalB was purified using the TALON™ metal affinity resin, fractionated on 12% Tris–glycine SDS–PAGE in the presence of 2 mM $CaCl_2$ or 2 mM EGTA, and stained with Coomassie blue. Molecular size markers (kDa) are indicated at the left. The mobility of CalB (marked with an arrow) was retarded in the presence of EGTA.

CaM [16], can be immobilized onto PVDF membrane (Fig. 4). Also, we found that a polyclonal serum prepared with biochemically purified CaM as antigen [17] cross-reacts with CalB (data not shown).

To document the Ca^{2+}-binding capacity of the protein, we used the modified $^{45}Ca^{2+}$ overlay technique of Maruyama [18]. Fig. 4 shows the binding of $^{45}Ca^{2+}$ to the cell lysates from CalB overexpressing cells and to the purified CalB protein. In addition, we used the Ca^{2+}-induced mobility shift assay on Tris–glycine SDS–PAGE. The mobility of CalB in this system is dependent on Ca^{2+}/EGTA pretreatment (Fig. 5). Interestingly, we were not able to detect any shift on Tris–Tricine PAGE.

4. Discussion

We report here the cloning and characterization in *Dictyostelium* of a novel member of the EF-hand-type protein family, CalB. It is closest to CaMs, although the degree of identity between CalB and any of the CaMs ($\sim$50%) is less than the degree usually observed within the CaM family, which is ca. 80%. The phylogenetic trees (All against All sequence-related peptide database, [25]) position CalB within CaMs and apart from myosin light chain and troponin C families ($<$30% identity).

We have also used three-dimensional coordinates of chicken CaM to construct an energy-optimized model of CalB structure (data not shown; ProModII, [26]). In contrast to CalB, primary sequence data of other small Ca^{2+}-binding proteins (CBP1, CBP2, CBP3 and calfumirin, [6–9]) do not fit the CaM template. Clearly, CalB is closest to CaM among the *Dictyostelium* proteins identified so far.

The deduced amino acid sequence of CalB, which is aligned in Fig. 2, shows some deviations from the CaM consensus. Whereas not all of the four potential Ca^{2+}-binding sites of CalB may bind Ca^{2+} in vivo, the protein may still function as a Ca^{2+} sensor. It is well established for CaM that the fractional occupancy of Ca^{2+}-binding sites required for activating a specific target can vary from one (phosphodiesterase activation) to four (activation of microtubule depolymerization) [23]. The occupancy of one site of CaM is sufficient to trigger conformational change of the protein [23]. We demonstrated that the electrophoretic mobility of CalB is Ca^{2+} sensitive, which is in contrast to the behavior of Ca^{2+} buffer proteins, such as parvalbumin [9].

Ca^{2+} signaling was documented in the regulation of *Dictyostelium* chemotaxis and aggregation. Later in development, the changes in cytosolic Ca^{2+} influence gene expression during the differentiation of prespore and prestalk cells (for review [1,2]). Interestingly, and in contrast to *Dictyostelium* CaM, CalB has a developmentally regulated expression pattern, reaching maximum levels during aggregation and in spores.

We inactivated the *calB* gene by insertion of the BsR selection cassette into its second exon. The mutant cells grow normally, develop and produce viable spores. It is our opinion that this finding may reflect the overlap of functions of closely related genes, although we cannot exclude the possibility that the first two EF-hand motifs of CalB could be produced in the mutants. It is unlikely, however, that the protein product of mutated *calB* plays any role in the *calB*$^-$ cells, because truncated CalB, containing only the first two EF-hand motifs, would not be able to act as a calcium-dependent regulatory protein.

It was soon assumed that CaM is the major intracellular Ca^{2+} receptor, also because many of its 'target' proteins were identified and their physiological roles established [23]. In contrast, many less abundant CaM-type proteins, including the proteins predicted in the genome projects, remain orphan. The assignment of these orphans, such as CalB, to their respective functions (and substrates) may advance our understanding of the diversity of Ca^{2+} signaling.

Acknowledgements: This work was supported in part by the Ministry of Education of Czech Republic (J13/98:11300003). We thank Prof. R.P. Dottin for the *Dictyostelium* cDNA library and Prof. M. Clarke for the helpful discussions and generous gifts of anti-CaM antibodies. The technical assistance of L. Fišerová is greatly acknowledged.

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