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Cloning and expression in *Escherichia coli* of a cDNA encoding a developmentally regulated Ca^{2+}-binding protein from *Dictyostelium discoideum*

Barrie Coukell*, John Moniakis, Avie Grinberg

Department of Biology, York University, 4700 Keele St., North York, Ont., M3J 1P3, Canada

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Abstract **We have cloned a full-length cDNA from *Dictyostelium discoideum* which encodes a new Ca^{2+}-binding protein. The deduced protein (termed CBP1) is composed of 156 amino acids and contains four consensus metal-ligating loop sequences found in helix-loop-helix motifs of many Ca^{2+}-binding proteins. When expressed in bacteria as a GST fusion protein, CBP1 binds Ca^{2+} in a $^{45}Ca^{2+}$ overlay assay. CBP1 exhibits little amino acid sequence homology with *Dictyostelium* calmodulin or calfumirin-1 (CAF-1) except in the putative Ca^{2+}-binding regions. Moreover, unlike calmodulin and CAF-1 expression, CBP1 mRNA is expressed preferentially during the multicellular stages of development.**

Key words: cDNA; Calcium-binding protein; Developmental expression; *Dictyostelium discoideum*

1. Introduction

During early development of *Dictyostelium discoideum*, the amoebae buffer the Ca^{2+} concentration of their external environment and take up substantial amounts of extracellular Ca^{2+} when stimulated by their natural chemoattractants, folate andcyclic AMP [1–3]. This accumulated Ca^{2+} is rapidly sequestered into a variety of organelles [4–6] and, during late development, most of it becomes localized in prestalk and anterior-like cells [7, 8]. Extracellular cyclic AMP also acts through specific cell surface receptors to mobilize Ca^{2+} from non-mitochrondial, IP_3-sensitive intracellular stores [9]. Ionic Ca^{2+} has been implicated in the regulation of a number of processes during development including protein secretion [10], cell aggregation [11, 12], cellular adhesion [13] and cell type-specific gene expression [14, 15]. Therefore, this ion might play a important role at certain stages of *Dictyostelium* development. To date, however, only a few Ca^{2+}-binding proteins have been characterized in this organism and most are expressed at relatively constant levels during growth and development [16, 17]. Consequently, it is unlikely that these proteins function to regulate specific developmental processes.

In this paper, we describe the cloning and characterization of a cDNA which encodes a new Ca^{2+}-binding protein (CBP1) in *Dictyostelium*. Interestingly, CBP1 mRNA is expressed at high levels only during the multicellular stages of development.

*Corresponding author. Fax: (1) (416) 736 5698.

The nucleotide sequence reported here has been submitted to the EMBL/GenBank database under the Accession Number X82784.

2. Materials and methods

2.1. Growth and development of amoebae

Dictyostelium discoideum strain AX2 was grown non-axenically with *Klebsiella aerogenes* on SM agar plates [18] or axenically in HL-5 medium [19] to a density of 5×10^6 cells/ml. The cells were washed free of bacteria or growth medium by centrifugation in SS [20] and permitted to developed on phosphate-buffered agar as described previously [21]. At the times indicated, amoebae were scraped from the plates, washed in SS and quickly frozen as pellets ($2–3 \times 10^7$ cells/tube) in dry-ice/ethanol.

2.2. cDNA cloning, sequencing and analysis

A 0.6 kb *Eco*RI fragment was isolated as a component of a chimeric insert while screening a *Dictyostelium* λgt11 cDNA library (provided by Dr. P. Devreotes, Johns Hopkins University, Baltimore, USA) prepared from cells starved for 3 h. This fragment was cloned into the *Eco*RI site of Bluescript II KS^+ vector (Stratagene), and sequenced. Analysis of this sequence revealed a single long open reading-frame and a partial 3′-untranslated sequence (UTS) but no translation initiation site. To obtain a full-length cDNA, the 0.6 kb fragment was used as a probe to screen a 16 h λgt11 cDNA library (obtained from Dr. M. Tasaka, NIBB, Okazaki, Japan) using standard methods [22]. Fifty-seven positive plaques were obtained from approximately 6×10^4 phage and the four strongest hybridizing phage were plaque purified. The largest insert (0.7 kb) obtained from these phage was cloned into the *Eco*RI site of the KS^+ vector to give plasmid pB0.7CBP, and the insert was sequenced. Double-stranded plasmid DNA was sequenced by the chain termination method [23] using universal and internal sequence-specific primers. DNA and amino acid sequence homology searches were carried out on the EMBL/GenBank database using the FASTA program. The structure of the deduced protein was analyzed with PROSIS ver.5.0 and amino acid sequence alignments were performed with the assistance of the program CLUSTAL.

2.3. Northern and Southern blot analysis

Total RNA was isolated from frozen cell pellets, size fractionated and transferred to nylon membranes as described previously [24]. The filters were probed with the 0.6 kb cDNA fragment labelled with ^{32}P by random priming. Hybridization and washing conditions were as described [25].

Genomic DNA was isolated from *Dictyostelium* AX2 cells as described [26]. For Southern blots, 3 μg of genomic DNA was digested with several restriction enzymes, size fractionated on a 0.7% agarose gel and transferred to nylon membranes. To provide high stringency conditions, the membranes were hybridized with the 0.6 kb probe in 1 M NaCl, 1% SDS, 10% PEG at 65°C and then washed twice for 5 min in 2 × SSC at room temperature (RT), twice for 30 min in 2 × SSC, 1% SDS at 65°C and twice for 30 min in 0.1 × SSC at RT. After exposure to X-ray film at −80°C, the membranes were stripped by boiling in 0.1 × SSC, 0.1% SDS for 30 min. For low stringency conditions, hybridization was at 45°C and the membranes were washed twice for 5 min in 2 × SSC at RT and twice for 30 min in 2 × SSC, 1% SDS at 45°C.

2.4. Expression of CBP1 in E. coli and $^{45}Ca^{2+}$ binding

To express CBP1 as a GST fusion protein, the full-length cDNA was amplified from pB0.7CBP by PCR and cloned into the vector pGEX-2T (Pharmacia/LKB). The sense primer(5′-AGGGATCCATGGATTGTGCAATTACA) corresponded to the first 6 codons of the coding region

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with a *Bam*HI restriction site on the 5′ end. The antisense primer (5′-CGGAATTCTCGAGATCTTTTTTTTTTTT) was a universal poly T primer with an *Eco*RI restriction site. The reaction (100 μl) contained 320 pmol of each primer, 20 nmol of each dNTP, 100 ng of pB0.7CBP DNA, 2 units of *Taq* DNA polymerase (Perkin-Elmer/Cetus), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$. Amplification was performed in a Perkin-Elmer/Cetus thermal cycler using a 'touchdown' protocol [27]. After an initial cycle of denaturation at 94°C for 1 min, annealing at 58°C for 2 min and extension at 72°C for 1 min, this was followed by cycles of 94°C/56°C/72°C (2 cycles), 93°C/54°C/72°C (2 cycles), 93°C/52°C/72°C (2 cycles), 93°C/50°C/72°C (25 cycles), and finally 72°C for 10 min. The single PCR product of ~0.65 kb was cloned in-frame into *Bam*HI/*Eco*RI-digested pGEX-2T vector (pGEX-*cbpA* construct), and transformed into *E. coli* DH5α (GIBCO/BRL).

After growth for 1 h at 37°C, *E. coli* transformants were further incubated with or without 200 μM IPTG for 7 h, washed once in PBS and frozen at −20°C. The GST-CBP1 fusion protein was isolated with glutathione-agarose beads [28] and CBP1 was cleaved by incubation with thrombin (200 μg/ml) in 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol, pH 8.0 for 1 h at 37°C.

$^{45}Ca^{2+}$ binding to crude bacterial extracts, purified CBP1 and bovine calmodulin (Sigma) was performed as described by Maruyama et al. [29]. Ruthenium red staining of Ca^{2+}-binding proteins was according to [30]. Protein concentration was determined by the method of Lowry et al. [31] using bovine serum albumin as a standard.

3. Results

During the isolation of cDNAs encoding P-type ion pumps in *Dictyostelium*, we cloned and sequenced a 0.6 kb DNA fragment which appeared unrelated to these ATPases. Analysis of this sequence suggested that it was a partial cDNA with a single long open reading-frame and a 3′-UTS but lacking a translation initiation site. A search of the EMBL/GenBank database with the deduced amino acid sequence failed to reveal any proteins with extensive homology to the sequence. However, specific regions of the sequence showed some homology to the 12 residue metal-binding loops of the helix-loop-helix motifs found in many Ca^{2+}-binding proteins [32]. Further examination revealed that the deduced protein possessed four such sequences; thus it was tentatively named CBP1 (calcium-binding protein-1) and the gene was designated *cbpA*.

To isolate a full-length cDNA, the 0.6 kb fragment was first used to probe a Northern blot of total RNA isolated from non-axenically grown cells at different stages of development (Fig. 1). The results revealed a single transcript of about 800 nt which is present at high levels in aggregation stage cells (~9 h) and through late development. During vegetative growth and early development, however, the level of this mRNA is very low. A similar pattern of expression is seen when the amoebae are grown axenically to mid-log phase before development (data not shown). Based on this pattern of expression, the partial cDNA was next used to screen a 16 h cDNA library, and the longest insert obtained (0.7 kb) was cloned and sequenced.

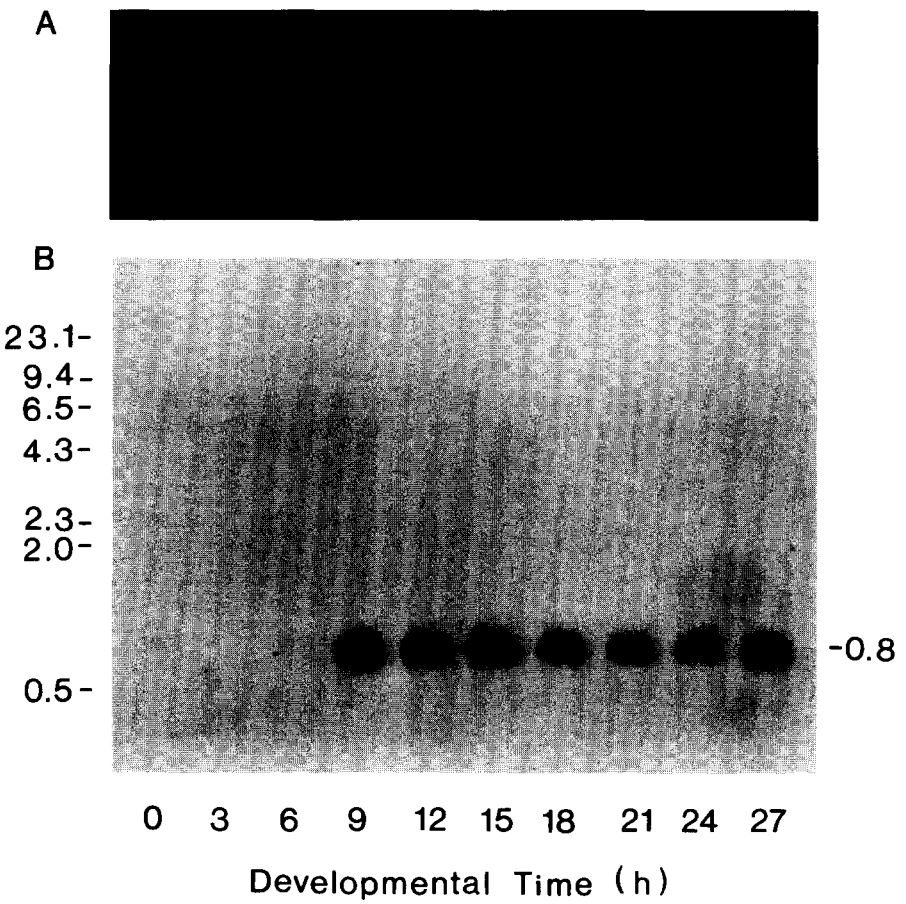

Fig. 1. Northern blot analysis of *cbpA* gene expression during *D. discoideum* development. Total RNA (20 μg/lane) isolated from non-axenically grown AX2 cells at different stages of development was fractionated on agarose/formaldehyde gels, stained with ethidium bromide to evaluate loading (A), transferred to nylon membranes and probed with the 0.6 kb cDNA (B). In B, molecular size markers (kb) are shown on the left while the estimated size of the transcript (knt) is indicated on the right. Stages of development: 0 h, vegetative cells; 9 h, aggregation; 15 h, tipped aggregates; 18 h, slugs and fingers; 21–24, culmination; 27 h, fruiting bodies.

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                                                        GTAAAATTAAAAA  13
                                                          *     *

    ATGGATTGTGCAATTACAAAAGATGTAGAGGATATGTTAAGAAAATTTGATTCAAATGGT  73
1    M  D  C  A  I  T  K  D  V  E  D  M  L  R  K  F  D  S  N  G
                                                    ----------

    GATGGTAATATTACATTTGATGAAGCTGTAAAACGTTTGAAGGAAACCGGTTCAAAAGAT 133
21   D  G  N  I  T  F  D  E  A  V  K  R  L  K  E  T  G  S  K  D
    --- 1 ----------------

    CCATTACGTGCTGCCTCTTCAATGTTTATTTCTCTTGATAAGGATAAGGATGGAATTATT 193
41   P  L  R  A  A  S  S  M  F  I  S  L  D  K  D  K  D  G  I  I
                                          -------------- 2 -----

    TCAATAAAAGAGATTCATGGCCATAAGGCTGATGTTGCTGCAAAGAAATTGCAAAAGGCA 253
61   S  I  K  E  I  H  G  H  K  A  D  V  A  A  K  K  L  Q  K  A
    ----------

    ATTAACAACATCTGTAACAACTTCTTGAAAGGCTATGACACTGATAAGGATGGAAGGATC 313
81   I  N  N  I  C  N  N  F  L  K  G  Y  D  T  D  K  D  G  R  I
                                          -------------- 3 -----

    TCATGGGATGAAGTATGCAATTGGGTAAATAAAAACAATCCAGATGCAATTGCTCCACTT 373
101  S  W  D  E  V  C  N  W  V  N  K  N  N  P  D  A  I  A  P  L
    ----------

    ATGATTGTTGAAAATTTCTTTAGTGAATTGGATAAAGATAATGACAGATTCGTAACCAAG 433
121  M  I  V  E  N  F  F  S  E  L  D  K  D  N  D  R  F  V  T  K
                                    ---------- 4 ---------

    TGTGAACTTCAGGAGTACGTTACTAAATACAAAAGTCTCCCAGAACAATAAATAATTTAT 493
141  C  E  L  Q  E  Y  V  T  K  Y  K  S  L  P  E  Q  *
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    TGTTCAAATTCAATAATTATTAATCAACATTTATAATTTTCATTTTTAAAAAAATTTAAA 553

    TAAATAAATAAATAAAAAATAATCTTTTAAAAATAAAAAAA                    594
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Fig. 2. Nucleotide and deduced amino acid sequence of the full-length *cbpA* cDNA. Nucleotides are numbered on the right and amino acids on the left. In-frame TAA stop codons are indicated by asterisks. The four consensus Ca^{2+}-binding sites (numbered dashed lines) and three potential polyadenylation signals (solid lines) are underlined. Only 110 nucleotides of the 3′-UTS are shown.

The sequences of the 0.6 kb and 0.7 kb cDNAs were identical in the overlapping region, but the new cDNA had a 20 bp extension on the 5′-end which included a consensus *Dictyostelium* translation initiation sequence (AAAATGG) and two upstream TAA stop codons in-frame. The nucleotide sequence and deduced amino acid sequence of the full-length cDNA is shown in Fig. 2. The open reading-frame encodes a very hydrophilic protein of 156 amino acids with a calculated molecular weight of 17,740 Da. Fifty-five of the 156 amino acids (35%) are charged with an overall net charge of −3. The four putative Ca^{2+}-binding sites are distributed evenly in the primary structure: site 1, residues 17–28; site 2, residues 53–64; site 3, residues 93–104; site 4, residues 131–142. Secondary structure analysis [33] predicts that these sequences reside in the loop regions of helix-loop-helix configurations.

Southern blot analysis of *D. discoideum* genomic DNA at high stringency suggests that *cbpA* is a single copy gene (Fig. 3, left). Reprobing the membrane at low stringency failed to reveal additional strongly hybridizing bands (Fig. 3, right). Therefore, the genome does not appear to possess other genes with a similar overall sequence.

To determine if CBP1 is an authentic Ca^{2+}-binding protein, it was expressed in *E. coli* as a GST-CBP1 fusion protein and assayed for $^{45}Ca^{2+}$ binding [29]. Fig. 4A, left, shows binding of $^{45}Ca^{2+}$ to extracts of cells carrying the pGEX-2T vector or the pGEX-*cbpA* construct grown with or without IPTG. The only radioactive band is in extracts of the strain harbouring the pGEX-*cbpA* plasmid induced with IPTG (lane 4). This band corresponds to a protein of ~44 kDa, the expected size of the fusion protein. Amido black staining of the membrane (Fig. 4A, right) shows that neither the 26 kDa GST protein alone (lane 2) nor the molecular weight markers (lane M), although present in similar amounts to the fusion protein, bind appreciable $^{45}Ca^{2+}$. When the fusion protein is isolated on glutathione-agarose beads and cleaved with thrombin, a single $^{45}Ca^{2+}$-binding protein of ~18 kDa is seen (Fig. 4B, left, lanes 3 and 4). Unlike bovine calmodulin (lanes 1 and 2), preincubating CBP1 with 20 mM EGTA or Ca^{2+} does not induce a mobility shift on SDS gels. Although calmodulin and CBP1 appear to bind similar amounts of $^{45}Ca^{2+}$, the stained membrane (Fig. 4B, right) shows that less calmodulin is retained by the nitrocellulose (see [34]). Therefore, recombinant CBP1 probably binds less Ca^{2+} than does calmodulin. Similarly, bacterially expressed CBP1 is

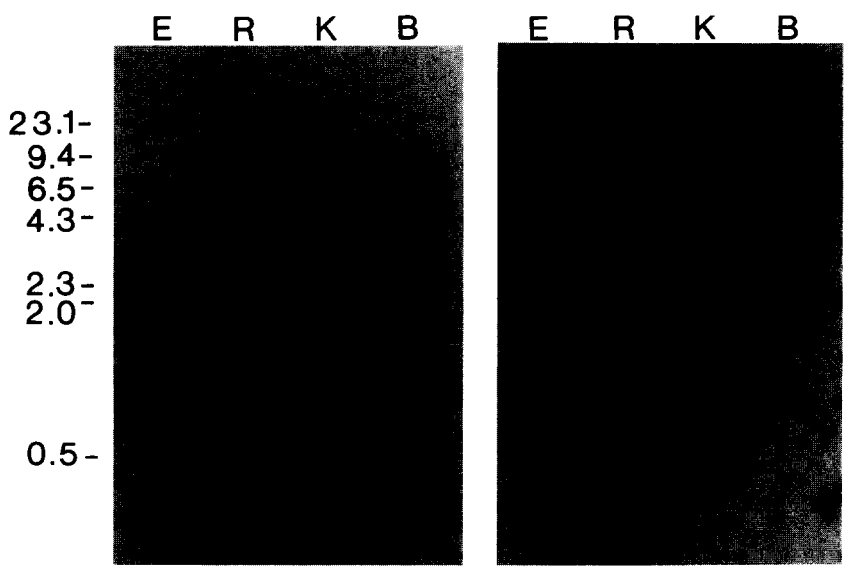

Fig. 3. Southern blot analysis of genomic DNA from *D. discoideum* strain AX2. DNA (3 μg) was digested with *Eco*RI (E), *Eco*RV (R), *Kpn*I (K) or *Bgl*II (B), fractionated, transferred to membranes and probed with the 0.6 kb cDNA. Membranes were hybridized and washed under high (left panel) or low (right panel) stringency conditions as described in section 2. Molecular size markers (kb) are indicated at the left of the figure.

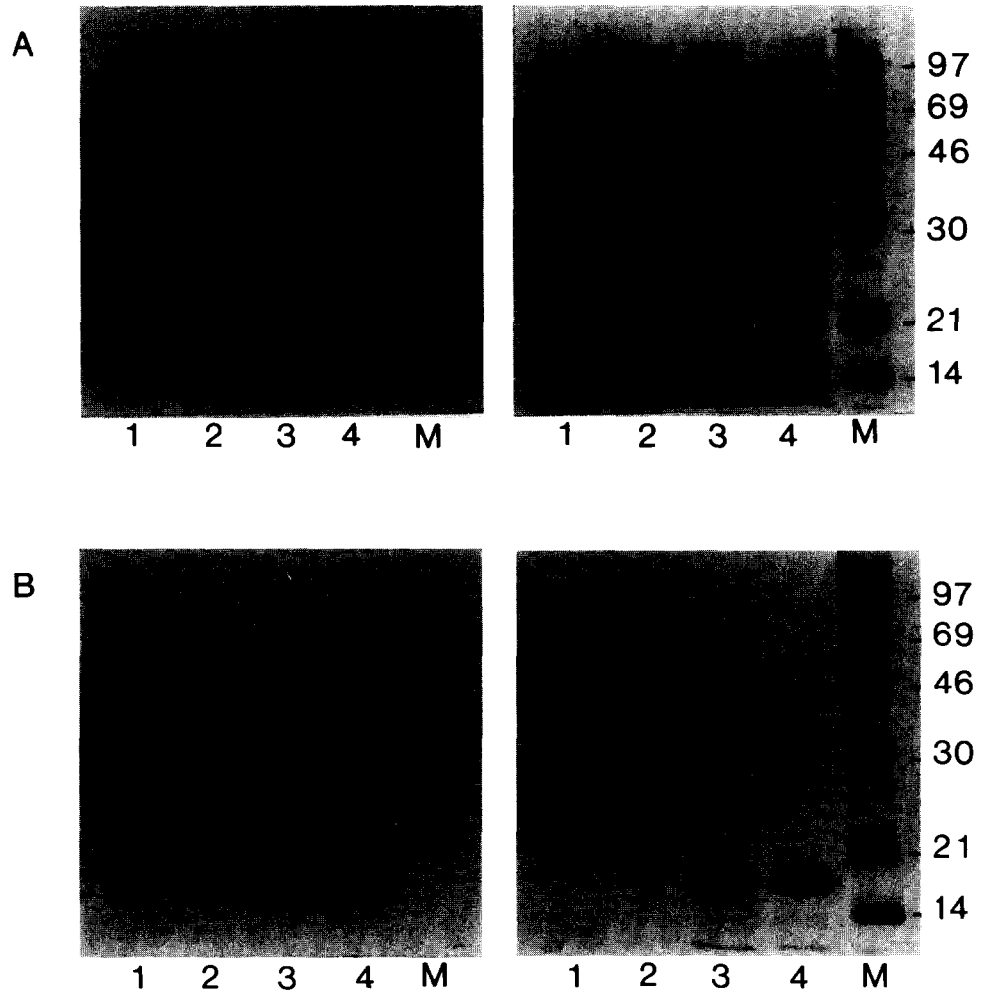

Fig. 4. Binding of $^{45}Ca^{2+}$ by CBP1. (A) *E. coli* cells carrying pGEX-2T (lanes 1 and 2) or pGEX-*cbpA* (lanes 3 and 4) were grown without (lanes 1 and 3) or with (lanes 2 and 4) IPTG as described in section 2. Solubilized cell extracts (30 μg protein/lane) were fractionated by SDS-PAGE on a 10% gel and transferred to nitrocellulose. (B) Two μg each of bovine calmodulin (lanes 1 and 2) and thrombin-cleaved CBP1 (lanes 3 and 4) were preincubated at RT for 30 min with 20 mM EGTA (lanes 1 and 3) or 20 mM Ca^{2+} (lanes 2 and 4), and then fractionated and transferred as described above. The membranes were first incubated with $^{45}Ca^{2+}$ (left) and then stained with amido black (right). The sizes (kDa) of the protein molecular weight markers (lane M) are indicated at the right of the figure.

less effective than calmodulin at binding ruthenium red (data not shown).

4. Discussion

In this paper, we provide molecular and biochemical evidence for a new Ca^{2+}-binding protein (termed CBP1) in *D. discoideum*. Certain structural features of CBP1, such as its small size and four putative ion-binding sites, resemble *Dictyostelium* calmodulin [35, 17] and calfumirin-1 (CAF-1), another small Ca^{2+}-binding protein recently identified in this organism [36, 37]. However, CBP1 differs from these other two proteins in a number of interesting ways. First, the three proteins have very different primary structures. Aligned pairwise, CBP1, calmodulin and CAF-1 exhibit ≤28% amino acid sequence identity, and muchof this homology is associated with the four putative Ca^{2+}-binding regions (Fig. 5). Second, *Dictyostelium* calmodulin (like most vertebrate calmodulins) and CAF-1 lack cysteine and tryptophan residues while CBP1 contains both amino acids (4 Cys and 2 Trp). Third, all three proteins are very hydrophilic with a large fraction (>35%) of charged amino acids. However, calmodulin is very acidic (net charge of −22) while CAF-1 is moderately acidic (net charge of −9) and CBP1 is only slightly acidic (net charge of −3). Finally, calmodulin is expressed relatively constitutively during *Dictyostelium* growth and development while CAF-1 mRNA is transcribed predominately during the first few of hours of development and CBP1 mRNA is present at high levels only after cell aggregation (Fig. 1). These distinct patterns of expression suggest that the three proteins also differ in function during development.

Recombinant CBP1 appears to bind Ca^{2+} less efficiently than bovine calmodulin (Fig. 4B), even though both proteins possess four consensus Ca^{2+}-binding sites. The reduced ability of CBP1 to bind Ca^{2+} could be due to subtle differences in the types of amino acids present at the critical ligand-binding positions of the Ca^{2+}-binding regions. However, this seems unlikely since the amino acids at most of these position are the same ones frequently found in high-affinity Ca^{2+}-binding sites of other proteins [32]. A more likely explanation for this result is that recombinant CBP1 is processed incorrectly in *E. coli* and it is

Fig. 5. Pairwise alignment of deduced amino acid sequences of *D. discoideum* calmodulin (CaM, 152 amino acid), CBP1 (156 amino acids) and CAF-1 (169 amino acids). Identical amino acids are indicated by an asterisk. Residues in the putative Ca^{2+}-binding loops are shown in bold type. Dashes denote gaps introduced into the sequence to maximize the alignment.

unable to assume the conformation necessary for optimal Ca^{2+} binding. This idea is supported by the observation that Ca^{2+} binding does not induce a shift in the mobility of the protein during SDS-PAGE (Fig. 4B, left). A detailed analysis of Ca^{2+} binding by CBP1 must await purification of the native protein.

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