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Cloning of a cDNA encoding a new calcium-binding protein from *Dictyostelium discoideum* and its developmental regulation[1]

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Abstract By employing 2D-PAGE, a protein differentially expressed during the development of *Dictyostelium discoideum* was discovered. The full cDNA of this protein was cloned using RT-PCR. The deduced protein is composed of 166 amino acid residues containing four EF-hand domains typical for calcium-binding proteins and was named CBP3. This protein shows little amino acid sequence homology with the other calcium-binding proteins from *D. discoideum* except EF-hand domains. The CBP3 mRNA was absent in vegetative amebas and accumulated maximally at 6 h of the development on filters. The mRNA level decreased thereafter and disappeared after 12 h of the development, while the protein level peaked at 8 h of development and remained constant thereafter. The mobility of CBP3 on SDS gel was shifted by treatment with EGTA, confirming the Ca^{2+}-binding activity of the protein.

Key words: Calcium-binding protein; EF-hand; Development; *Dictyostelium discoideum*

1. Introduction

Calcium ion (Ca^{2+}) is believed to mediate a variety of cellular processes as a second messenger in signal transduction. The roles of Ca^{2+} are mediated by a variety of calcium-binding proteins, which act as buffers storing and releasing Ca^{2+}, or as sensors detecting Ca^{2+} concentration and transducing the signals [1]. Calbindin and calmodulin are the representative calcium-binding proteins that perform such buffering roles and sensing roles, respectively. Most calcium-binding proteins possess characteristic calcium-binding regions called EF-hand domains [2].

The cellular slime mold *Dictyostelium discoideum* is an excellent organism for research on cell differentiation and development. The role of Ca^{2+} in the regulation of development of *D. discoideum* is being widely studied and many lines of evidence indicate that Ca^{2+} is involved in numerous processes, such as chemotactic signalling [3–5], cell-cell adhesion [6], cell fate determination [7–10], and transduction of starvation signals [11]. Several EF-hand-type calcium-binding proteins have also been reported in *D. discoideum*, such as calmodulin [12], calfumirin-1 [13], CBP1 [14], and CBP2 [15]: these proteins have similar properties in that they have small sizes of about 20 kDa and the sequences of EF-hand domains and their relative positions in primary structures are almost the same. However, they show different expression patterns during development. Calmodulin is expressed constitutively during growth and development [12] and calfumirin-1 is mainly produced during transition from growth to development [13]. On the other hand, CBP1 and CBP2 are expressed after cell aggregation [14,15].

Here, we report the discovery of a new calcium-binding protein in this organism and the differential expression of this protein during development. As the exact function of this protein is not clear at present, we gave the temporary name CBP3 to the protein following CBP1 and CBP2 that have been reported previously [14,15].

2. Materials and methods

2.1. Growth and development of D. discoideum

An axenic strain of *D. discoideum* (AX3) was grown axenically in HL5 medium at 22°C. For development, cells were harvested at a density of $3–5\times10^6$ cells/ml, washed twice with development buffer (DB) (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$) and deposited on the surface of non-nutrient agar plates (DB, 1.5% agar) [16].

2.2. Two-dimensional gel electrophoresis and protein sequencing

Harvested cells were suspended in 20 mM Tris-HCl (pH 7.5) containing protease inhibitors and broken by bead beating. After removing debris by brief centrifugation, the cell extract was concentrated by mixing with the same volume of 20% trichloroacetic acid in acetone. The protein pellet was washed with cold acetone, dried on air and dissolved in the buffer recommended by O'Farrell [17]. Preparation of rod gel and isoelectric focusing were carried out according to the method proposed by O'Farrell [17]. About 1 mg of protein equivalent to 4×10^7 cells was loaded on a rod gel. Ampholine pH 5–8 (Amersham Pharmacia Biotech) and Bio-Lyte pH 3–10 (Bio-Rad) were mixed 1:1 and used as ampholytes. Cathode solution and anode solution were 25 mM NaOH and 25 mM H_3PO_4, respectively. After focusing, rod gel was subjected to 10% SDS-PAGE and proteins were visualized by Coomassie blue staining. For N-terminal sequencing, the proteins in the secondary slab gel were electrotransferred to polyvinylidene difluoride membranes (Millipore) essentially as described [18], except for using 10 mM CAPS buffer (pH 11) containing 10% methanol as transfer buffer. The protein spots were cut out and applied to a Procise Protein Sequencing System (Applied Biosystems).

2.3. cDNA cloning

cDNA was amplified by RT-PCR essentially as described [19]. First-strand cDNA was prepared by primer extension using primer dTT1 (5′-TCTCCGAATTCTAGATTTTTTTTTTTTTT-3′) and M-MLV reverse transcriptase. 4 μg of total RNA extracted from the cells developed for 8 h was used as a template in 50 μl reaction. PCR reaction was carried out using primer dTT2 (5′-TCTCCGAATTCTAGATTTTT-3′) and sense primer (5′-ATGTTNACHAAYAAYGARATYTAYCA-3′, where N = A+T+G+C, H = A+T+C, Y = T+C, R = A+G) corresponding to the N-terminal sequence MLTNNEIYQ. Initial denaturation for 3 min at 94°C, 40 cycles for 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and final extension for 10 min at 72°C were performed using a GeneAmp PCR system (Perkin Elmer). The PCR product was cloned into pGEMTeasy (Promega) and the sequence was determined on both strands. The sequence of the 5′-end region was obtained using a 5′-RACE kit (Boehringer Mannheim).

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[1]The nucleotide sequence reported here has been submitted to the GenBank/EMBL data bank under accession number AF076972.

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2.4. Northern and Southern blot analyses

Total cellular RNA was extracted from both vegetative amebas and cells developed for indicated times as described [20]. 20 μg of total RNA was size-fractionated on 1.2% agarose gel containing formaldehyde and transferred to nylon membrane by capillary blotting. 8 μg of genomic DNA extracted as described [21] was digested with restriction enzymes and electrophoresed on 0.6% agarose gel and transferred to nylon membrane. A digoxigenin (DIG)-labeled cDNA was used as a probe. Both hybridization and detection were carried out using a DIG DNA labeling and detection kit (Boehringer Mannheim) according to the manufacturer's recommendation.

2.5. Immunological methods

For the preparation of immunogen for polyclonal anti-CBP3 antiserum, CBP3 was overexpressed in *Escherichia coli* using histidine-tagging overexpression vector pET15b and host *E. coli* BL21 pLysS (DE3) strain (Novagen). After purification and removing histidine tag by thrombin treatment, the CBP3 was electrophoresed on 12% SDS-polyacrylamide gel and the protein-containing gel slice was homogenized. Four mice were immunized by injecting CBP3-containing gel homogenate, 20 μg of CBP3 per mouse, three times at intervals of 2 weeks. For Western blot analysis, total protein was fractionated by 12% SDS-PAGE and electrotransferred onto nitrocellulose membrane [18]. As a secondary antibody, anti-mouse IgG-alkaline phosphatase conjugate (Sigma) was used at 1:15 000 dilution.

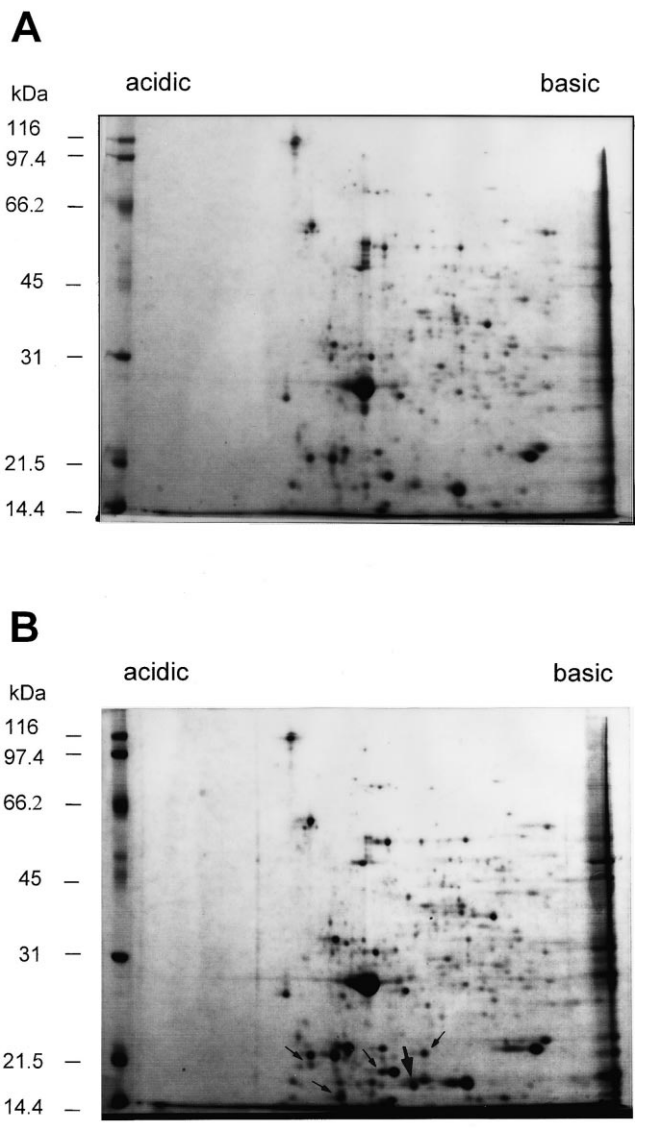

Fig. 1. Comparison of two-dimensional protein profiles. A: Vegetative amebas. B: 8-h developed cells. About 1 mg of total proteins for each was subjected to 2D-PAGE. The spots marked with arrows were subjected to N-terminal amino acid sequencing. The spot marked with a large arrow was the only protein that yielded N-terminal amino acid sequence and was studied in this paper.

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          CTTCAAATTCTTTTTTATTTTTGTAGTAAAAATTTACATATTTTAATATTACA   53

    ATGTTAACTAATAATGAAATTTATCAAGAAGTTCAAAAATTTGCCAAGGGTTATGACTTA  113
1     M  L  T  N  N  E  I  Y  Q  E  V  Q  K  F  A  K  G  Y  D  L

    AATCAAGATGGCGTATTAACTTCACAAGAAATATATTATTCACTTCTAAAAAAAATGAAT  173
21    N  Q  D  G  V  L  T  S  Q  E  I  Y  Y  S  L  L  K  K  M  N

    GGCAATTCTTATGAGGCATCAAAAGCAACTGGTGTTTTATGTTCAACAATTGATATAAAC  233
41    G  N  S  Y  E  A  S  K  A  T  G  V  L  C  S  T  I  D  I  N

    AAAGATGGTAAATTCAGTTATCATGAAATTGCTAAATATTGTGCTGACAATGCAAAAAAA  293
61    K  D  G  K  F  S  Y  H  E  I  A  K  Y  C  A  D  N  A  K  K

    CTAATTGAACAAAATGCTGATATTGCTGCATTGGCTGATGTTGAAGCTTTTTTATTAAGA  353
81    L  I  E  Q  N  A  D  I  A  A  L  A  D  V  E  A  F  L  L  R

    TTTGATAAAGATAAGGATAGAAAATTAAACAAAACAGAATTTGTTGAATACTTCAAAGGA  413
101   F  D  K  D  K  D  R  K  L  N  K  T  E  F  V  E  Y  F  K  G

    GGAACAGATACCCCATACTCTGATCGTGATTATGTTCTTAAAATTATTGATTTGGATAAA  473
121   G  T  D  T  P  Y  S  D  R  D  Y  V  L  K  I  I  D  L  D  K

    GATGGATGTGTTTCCGCAAATGAATTACAAGAATGGTTCAAAAAAAAAAGAATTGATTAT  533
141   D  G  C  V  S  A  N  E  L  Q  E  W  F  K  K  K  R  I  D  Y

    GCAAGCCGTCCACATTGTTAAAAAACTTGGGACATAATACCACATTAATCTTTTTTTTAA  593
161   A  S  R  P  H  C  *

    GGTTTGGTACATAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequence of *cbpC* cDNA. Four EF-hand calcium-binding regions are shown in bold. The stop codon is marked with an asterisk.

3. Results and discussion

We employed the 2D-PAGE technique to search for developmentally regulated proteins from *D. discoideum*. Total proteins from vegetative amebas, 8-h developed cells and 24-h developed cells were subjected to 2D-PAGE. For ease of later N-terminal sequencing, the gels were stained with Coomassie blue, in order that only the relatively abundant proteins could be visualized. As a result, some differences were noticeable between the protein profiles of vegetative amebas and 8-h developed cells, while there was no marked difference between the protein profiles of 8-h developed cells and 24-h developed cells. Fig. 1 shows the different protein expression patterns between the vegetative amebas and 8-h developed cells. From protein spots differentially expressed, five spots of small sizes were cut out and subjected to N-terminal amino acid sequence analyses. Only one of these proteins, which was estimated by the spot position in the 2D gel to have a molecular mass of about 20 kDa and isoelectric point of 5–6, yielded a sequence, MLTNNEIYQE. A redundant primer was designed from this sequence and used for RT-PCR. An amplified product of 560 bp was obtained from RT-PCR. In order to get the 5′-end region and confirm the sequence of N-terminal region from which the redundant primer was designed, we performed 5′-RACE. Finally, the full-length cDNA of 610 bp was cloned and sequenced.

The nucleotide sequence and the deduced amino acid sequence of this gene is shown in Fig. 2. The cDNA had a 53-bp extension at the 5′-end and the sequence surrounding the ATG start codon (TACAATGT) was somewhat different from a consensus translation initiation sequence (AAAATGG) of *D. discoideum* [22]. Translation of the sequence yielded a small protein of 166 amino acid residues, of which the molecular mass and isoelectric point were calculated to be

19 kDa and 5.28, respectively. These values agree well with the approximate estimation of molecular mass and isoelectric point from the spot position in the gel.

BLAST analysis revealed that this protein is similar to several small calcium-binding proteins identified in *D. discoideum*, such as calmodulin, calfumirin-1, CBP1, and CBP2 in primary structure. Four EF-hand domains, the characteristic feature of calmodulin-type calcium-binding proteins, were found in the sequence of this protein. Schleicher et al. [15] have suggested that *D. discoideum* contains a family of calmodulin-related proteins and described their new calcium-binding protein as CBP2. Following this suggestion, we named this protein CBP3 and the gene *cbpC* according to the order of discovery. The amino acid sequence of CBP3 was compared with those of these EF-hand proteins using the GCG program and their multiple alignment is shown in Fig. 3. As can be seen in the alignment, the EF-hand domains mainly have sequence homology between them and there is little sequence similarity in sequences other than the EF-hand domains. Some distinct features were found in their primary structures. All of these proteins have a large fraction of charged amino acids and the net charges of calmodulin (−22), calfumirin-1 (−9), and CBP1 (−3) were calculated previously [14]. In contrast to the highly acidic properties of calmodulin and calfumirin-1, CBP3 as well as CBP2 is nearly neutral (net charge of −1). Also, CBP3 contains four cysteine residues like CBP1, while CBP2 has one cysteine, and calmodulin and calfumirin-1 lack cysteine. When aligned pairwise, CBP3 shows 21% amino acid sequence identity to calmodulin, 30% identity to calfumirin-1, 28% identity to CBP1, and 25% identity to CBP2. Further homology searching has failed to give any other proteins that have significant sequence homology to CBP3. Many other EF-hand calcium-binding proteins from various sources showed extremely low similarity to CBP3 and they have some sequence homology only in the EF-hand regions.

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CBP3  -------MLTNNEIYQEVQKFAKGYDLNQDGVLTSQEIYYSLLKKMNG   41
CaM   ---MASQESLTEEQIAEFKEAFSLFDKDGDGSITTKELGTVM---RSL   42
CAF1  -------MASTQNIVEEVQKMLDTYDTNKDGEITKAEAVEYF---KGK   38
CBP1  ---------MDCAITKDVEDMLRKFDSNGDGNITFDEAVKRL---KET   36
CBP2  MSATVHYKDIRKGMEKDLESLFKKYDSDRNGKITYIEIVETL---RKA   45

CBP3  NSYEASKATGVLCSTIDINKDGKFSYHEIAKYCA-------DNAKKLI   82
CaM   GQNPTEAELQDMINEVDADGNGNIDFPEFLT-------------MMA   76
CAF1  KAFNPERSAIYLFQVYDKDNDGKITIKELAGDIDFDKALKEYKEKQAK   86
CBP1  GSKDPLRAASSMFISLDKDKDGIISIKEIHGHKA-----------DVA   73
CBP2  GKKNPERIA-DLLFRDDTDKNGELTIEEAKLRIV-----------RMN   81

CBP3  EQNADIAALADVEAFLLRFDKDKDRKLNKTEFVEYF---KGGTDTEYS  127
CaM   RKMQDTDTEEEIREAFKVFDKDGNGYISAAELRHVM--TSLGEKLTNE  122
CAF1  SKQQEAEVEEDIEAFILRHNKDDNTDITKDELIQGF--KETGAKDPEK  132
CBP1  AKKLQKAINNICNNFLKGYDTDKDGRISWDEVCNWVNKNNPDAIAPLM  121
CBP2  DEKIEKVLNWDVEKFINDNDKDGDRKITRDEVLQRF--TEQGAEDPEL  127

CBP3  DRDYVLKIIDLDKDGCVSANELQEWFKKKRIDYASRPHC--  166
CaM   EVDEMIREADLDGDGQVNYDEFVKMMIVRN----------  152
CAF1  SANFILTEMDTNKDGTITVKELRVYYQKVQ----KLLNPDQ  169
CBP1  IVENFFSELDKDNDRFVTKCELQEYVTKYK------SLPEQ  156
CBP2  ITDSIFRQMDLDRDGVITCDEIKEFNRKKKFSFLKSSAPKQ  168
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Fig. 3. Comparison of the amino acid sequence of CBP3 with other small EF-hand calcium-binding proteins from *D. discoideum*. The amino acid sequences of calmodulin (CaM) [12], calfumirin-1 (CAF1) [13], CBP1 [14], and CBP2 [15] were aligned for maximal similarity with the CBP3 sequence using the GCG program. Identical and similar residues are marked by shading.

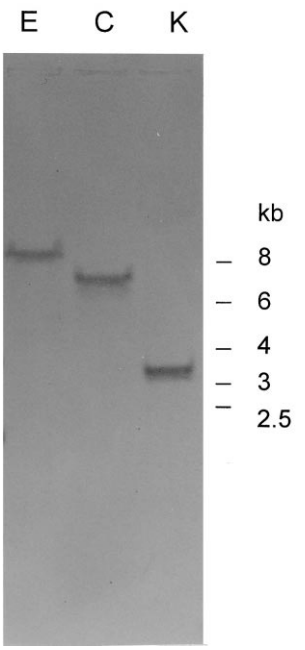

Fig. 4. Southern blot analysis of the genomic DNA of *D. discoideum*. 8 μg of genomic DNA was digested with *Eco*RI (E), *Cla*I (C), and *Kpn*I (K), electrophoresed, transferred to nylon membrane, and probed with DIG-labeled 0.6 kb cDNA.

Genomic Southern blot analysis was performed using the cDNA probe after digestion of genomic DNA with *Eco*RI, *Cla*I, and *Kpn*I (Fig. 4). Under high stringency, only one band was detected in all three lanes, indicating that a single gene encoding CBP3 exists in the genome of *D. discoideum*.

To reveal the developmental regulation of CBP3 expression, we investigated the mRNA and protein levels during the development of this organism. Total RNA and proteins were extracted from the cells at specific developmental stages, and applied to Northern and Western blot analyses (Fig. 5). As shown in Fig. 5A, the CBP3 mRNA was not detected in the vegetative amebas cultivated in HL5 medium. The mRNA was detected after 4 h of starvation in DB and its level peaked at 6 h of development, which corresponds to the early aggregation stage of development. Thereafter, the mRNA level declined and it was negligible after 12 h of development. However, as shown in Fig. 5B, the protein level of CBP3 reached a peak at 8 h and was maintained thereafter to be rather constant throughout the whole developmental stages. These facts mean that CBP3 is very stable against proteolytic degradation, enough to be maintained even after the protein production stops. From these facts, CBP3 is presumed to play a role not only specifically at the aggregation stage of development, but also at the subsequent stages after aggregation. Small calcium-binding proteins discovered so far in *D. discoideum* show diverse expression patterns during the course of vegetative growth and multicellular development. Calmodulin was expressed constitutively during growth and development [12], while calfumirin-1 was mainly produced during transition from growth to development [13]. The CBP1 mRNA was not produced until the cells were developed for 9 h and the expression was maintained at a rather constant level from 9 h to the end of development [14]. The CBP2 mRNA expression began at 9 h, reached a maximum at 12 h and stopped after

15 h of development [15]. As described above, calcium-binding proteins including CBP3 are expressed at different stages of development in *D. discoideum*, suggesting that they have different roles of their own at various stages of development. And it is probable that many other EF-hand type calcium-binding proteins, besides the above-mentioned proteins, are present and play a specific role in the growth and the development of this organism.

Calmodulins from various sources have a characteristic Ca^{2+}-binding property that the mobility of the protein in SDS-PAGE increases or decreases by preincubation with Ca^{2+} or EGTA, respectively [23]. To confirm the ability of CBP3 to bind Ca^{2+}, the mobility shift test was performed. Total proteins of 8-h developed cells were electrophoresed on a 12% SDS-polyacrylamide gel in the presence of 2 mM $CaCl_2$ or 2 mM EGTA and CBP3 detection was performed by Western blot analysis. As shown in Fig. 6, CBP3 exhibited different mobility in the gel depending on whether Ca^{2+} was bound or not. CBP3 did not show mobility shift after treatment with 2 mM Ca^{2+}, indicating that CBP3 exists mainly in Ca^{2+}-bound form in the cells (Fig. 6, lanes 1 and 2), while

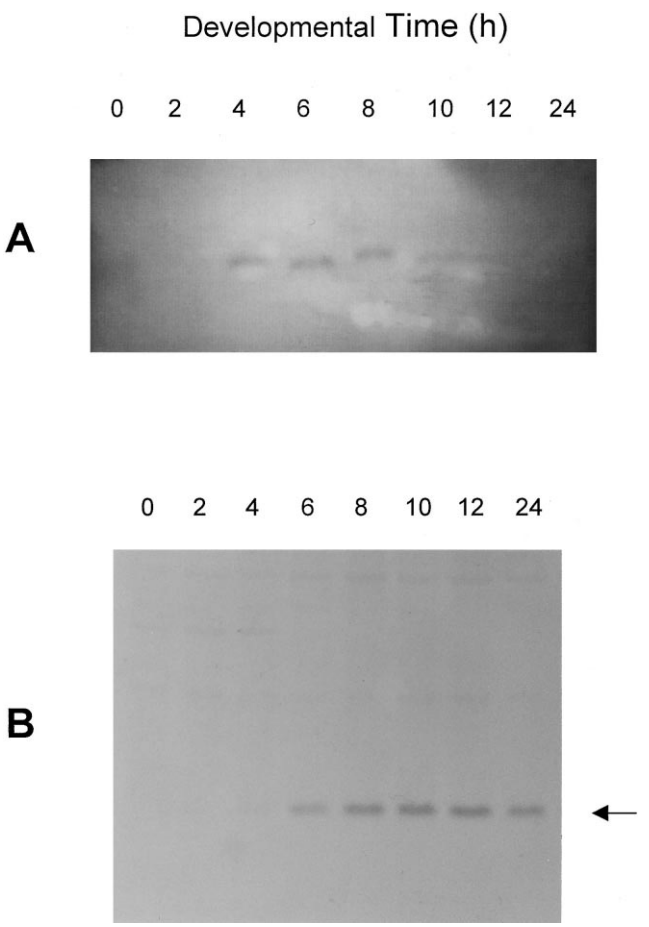

Fig. 5. Developmental regulation of CBP3 expression. A: Northern blot analysis of *cbpC* gene expression. Total RNA was extracted at the indicated developmental times. 20 μg of total RNA was fractionated on formaldehyde-containing agarose gel, transferred to nylon membrane and probed with DIG-labeled cDNA. B: Western blot analysis. 20 μg of total proteins isolated at different stages of development was fractionated on 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and incubated with polyclonal mouse antiserum raised against CBP3. The CBP3 is marked with an arrow on the right.

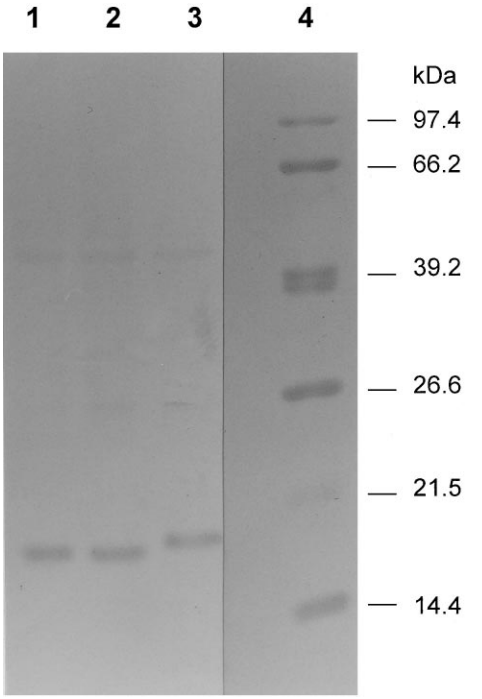

Fig. 6. Effect of calcium binding on the electrophoretic mobility of CBP3. The total protein extracted from 8-h developed cells was used as a reference without the addition of both $CaCl_2$ and EGTA (lane 1). The experimental condition was the same as in Fig. 5B, except that 2 mM $CaCl_2$ (lane 2) or 2 mM EGTA (lane 3) was added to the total protein used as a reference. Protein size markers (lane 4) stained with amido black are indicated on the right.

CBP3 migrated in the SDS gel more slowly in the presence of EGTA (Fig. 6, lane 3). On the other hand, the recombinant CBP1 and CBP2 expressed in *E. coli* did not exhibit a mobility shift even when they were preincubated with $CaCl_2$ or EGTA at high concentrations (20 mM in the case of CBP1). However, they only showed lower Ca^{2+}-binding activity than calmodulin, when tested by the $^{45}Ca^{2+}$ overlay method [14,15]. In the case of CBP2, the mobility shift was also not observed in the native protein. This means that both CBP1 and CBP2 have a low affinity for Ca^{2+}. Considering that many calcium-binding proteins acting as Ca^{2+} buffers generally have a low affinity for Ca^{2+}, it is probable that CBP1 and CBP2 have a function of Ca^{2+} buffering. Otherwise, CBP3 showed a high affinity for Ca^{2+}, like calmodulin but in contrast to CBP1 and CBP2. In this respect, CBP3 is different from CBP1 and CBP2 and rather similar to calmodulin and the function of CBP3 is also presumed to be homologous to calmodulin, acting as a Ca^{2+} sensor. Calmodulin and troponin C, well-known EF-hand proteins acting as Ca^{2+} sensors, are symmetrical dumbbell-shaped molecules separated by an α-helical structure and they undergo Ca^{2+}-induced conformational changes to activate their downstream targets [24]. However, calbindin, a calcium-binding protein known to act as a Ca^{2+} buffer, does not undergo such a conformational change [25]. So, the structures of Ca^{2+}-bound and Ca^{2+}-free CBP3 should be analyzed to elucidate the exact function of the protein.

Here, we report the isolation of cDNA encoding a new EF-hand calcium-binding protein and the developmental regulation of the protein expression. Among EF-hand proteins from

D. discoideum, calmodulin is the first to be identified and characterized extensively [26]. Many lines of evidence about the roles of calmodulin have been accumulated and indicate that calmodulin is involved in the regulation of a variety of cellular processes. Besides the studies about Ca^{2+}/calmodulin-dependent protein kinase [27] and Ca^{2+}/calmodulin-dependent protein phosphatase (calcineurin) [28], there have been many reports demonstrating the presence of a number of specific calmodulin-binding proteins in *D. discoideum* [29,30]. Recently, Maeda et al. [31] reported that the overexpression of calfumirin-1 has a stimulatory effect on differentiation, however, the protein is not essential for differentiation. The roles of CBP1 and CBP2 have not been presented yet, except for reports on their genes [14,15]. The low sequence homology, several characteristic features in their amino acid compositions, the difference in affinity for Ca^{2+}, and the different expression patterns of these small calcium-binding proteins may reflect the distant relationship between them, the different mechanisms of function, and their diverse roles during growth and development. To elucidate the precise functions and roles of CBP3 during development of this organism, we will focus on researches about the effects of overexpression or inactivation of the gene on developmental processes, structural analysis of the protein, and search for CBP3-binding proteins.

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