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EF-hands in CBP7 are Important in the Process of Development

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Abstract

Calcium ions play an important role in development and intracellular signaling. *Dictyostelium discoideum* has 14 genes encoding calcium-binding proteins (CBPs), but the function of most CBPs during development has not yet been studied. In this study, we investigated the specific functions of CBP7, one of 14 CBPs, in development using RNA interference cell lines of CBP7, cell lines overexpressing CBP7, cell lines with point mutations in the EF-hand domain, and cell lines expressing fragment proteins. was intended to reveal. CBP7 consists of 169 amino acids and contains 4 EF-hand domains. The CBP7-overexpressing cells showed complete loss of developmental process. These cells remained in the single-cell growth stage under development-inducing conditions, while wild-type cells formed aggregations within 6-8 h of development and eventually formed fruiting bodies. The experiments using point-mutated CBP7 protein showed that all EF-hand domains of CBP7 were important for CBP7 to function during developmental process. These results suggest that CBP7 plays an important role in developmental processes across all EF-hand domains.

Keywords: CBP7, Development, *Dictyostelium*, Calcium

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1. Introduction

The social ameba *Dictyostelium discoideum* has been used as a model system for investigating signaling pathways in eukaryotes[1]. Upon starvation, *Dictyostelium* amebae come together and begin a multicellular developmental process, which can be divided into

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aggregation and post-aggregation stages. The role of calcium ions in regulation of this developmental process has been widely studied in *Dictyostelium*. It has been shown that *Dictyostelium* regulates intracellular calcium concentrations during early development. The rate of Ca^{2+} influx is stimulated by the chemoattractant cAMP[2].

Calcium ions are required for the regulation of a variety of cellular processes and intracellular signaling, such as the chemotaxis, development, and differentiation[3]. Calcium ion concentrations are regulated by calcium-binding proteins (CBPs), either directly or indirectly. These proteins are deeply involved in the regulation of cytosolic and intracellular calcium concentrations, including calcium transport and calcium buffering. Many CBP proteins function as calcium sensors of calcium-induced conformational changes and transduce calcium signals to downstream effectors[4].

In previous studies, 14 different genes encoding small CBPs were identified in *Dictyostelium*. In a previous study, 14 different genes encoding small CBPs were identified in *Dictyostelium*. Among them, we investigated the phenotypes of cells expressing CBP7 or RNAi constructs in morphogenesis, growth, and development to understand the function of CBP7 in various biological processes. Results indicate that it is involved in the regulation of morphogenesis, growth and development.

2. Materials and Methods

2.1. Strains and cell culture

The *Dictyostelium* KAx-3 strain was cultured axenically in HL5 medium at 22 ˚C. The expression plasmids included green fluorescent protein (GFP)-CBP7. Transformants were maintained with 20 μg/mL G418. The coding sequence of CBP7 cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) with the tj66 and tj67 primers. The amplification fragment was ligated into the *EcoR*I-*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. Expression mutants CBP7N24A, D26A, CBP7D63, 65A, CBP7D117, 19A, and CBP7D152, 154A were generated by PCR using as the primers tj171 and tj172 for CBP7N24A, D26A; tj169 and tj170 for CBP7D63, 65A; tj143 and tj144 for CBP7D117, 19A; tj145 and tj146 for CBP7D152, 154A. To obtain the CBP7D117, 119,152,154A mutant,

CBP7D117, 19A was used as the template and tj145/tj146 as the primers. The resultant point-mutagenesis fragments were ligated into the *EcoR*I-*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. For the expression of truncated CBP7 proteins, the EF-hand 3, 4 deletion of the CBP7 sequence was generated by PCR using the full-length CBP7 cDNA coding sequence as the template and tj66 and tj151 as the primers. The EF-hand 1, 2 deletion of the CBP7 sequence was also constructed by PCR using the full-length CBP7 cDNA coding sequence as the template and tj168 and tj67 as the primers. All clones were confirmed by DNA sequencing. The amplification fragment was ligated into the *EcoR*I-*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. For making RNA interference construct, the full-length CBP7 fragment was synthesized using primer tj66, MR4 and cloned into pDAX-3H vector. The plasmid that had a sense orientation was digested with *EcoR*I. The short 5'-fragment of CBP7 was synthesized using primer MR5, tj67 and cloned into tj66/MR4 cloned vector.

Table. 1. PCR primer sequences used in this study.

Primer	sequence	Location
	5'→3' directed PCR primers	
tj66	ATG AGC ACT TGT GGT GAT AAT AG	1 - 23
tj143	GAC TTG ATA AAG CTA AGG CTA AAA AAT TAA ACA AAA CAG	309 - 348
tj145	CTT AAA ATT ATT GAT TTG GCT AAA GCT GGA TAT GTT TCC	405-444
tj168	GCT GCA TTG GCT GAT GTC GAA G	275 - 297
tj169	GTT CAA CAG TTG ATA TGG CTA ATG CTG GTA AAT TCA G	170 - 207
tj171	CCA AGA TTA TGA CTT AGC TAA GGC TTA CAG TGT AAC TTC	54 - 93
MR5	GCA GCT GGT GTT TTA TGT TCA	134 - 155
	3'→5' directed PCR primers	
tj67	TTA ACA AAT TGG ACC TCT TGC	490 - 511
tj144	CTC TTT TGT TTA ATT TTT TAG CCT TAG CTT TAT CAA GTC	309 - 348
tj146	GGA AAC ATA TCC AGC TTT AGC CAA ATC AAT AAT TTT AAG	405-444
tj151	AGT CTC AGC ATT TTG TTC AAT TTG	250 - 274
tj170	CAA GTT GTC AAC TAT ACC GAT TAC GAC CAT TTA AGT C	170 - 207
tj172	GCT TCT AAT ACT GAA TCG ATT CCG AAT GTC ACA TTG AAG	54 - 93
MR4	TTA ACA AAT TGG ACC TCT TGC	490 - 511

2.2. Fluorescence image acquisition

A hole was made in the center of a 3 ml plate and a slide glass was attached to the bottom. Vegetative Cells were allowed to attach to the plate for 15 min. Time-lapse fluorescence movies were taken using a microscope. The frames were captured by the NIS-Elements software (Nikon), and the movement of fluorescent cells was traced and analyzed using the Image J software (National Institutes of Health, USA).

2.3. Development

Growing cells were harvested, washed twice with 12 mM Na/K phosphate buffer (pH 6.1), and plated on Na/K phosphate agar plates at a density of 3.5×10^7 cells/mL. Development was examined at

various concentrations of $CaCl_2$ (0-50 mM) and ethylenediaminetetraacetic acid (EDTA; 0-6 mM). The developmental morphology of the cells was examined by photographing developing cells under a phase-contrast microscope.

3. Results

3.1. Identification of the gene encoding CBP7

There are 14 genes encoding CBP proteins in the *Dictyostelium* genome sequence (Table 2). Among them, CBP1, CBP3, CBP4a, and CBP4b have been characterized, and their functions have been studied in various biological processes (Table 2). However, the other CBP proteins have not been studied yet. Here, I characterized one of the 14 CBP proteins, CBP7. *Dictyostelium* CBP7 has 169 amino acids (expected molecular mass of 19.3 kDa), EF-hands 1 and 2 at the N-terminal region and EF-hands 3 and 4 at the C-terminal region (Fig. 1).

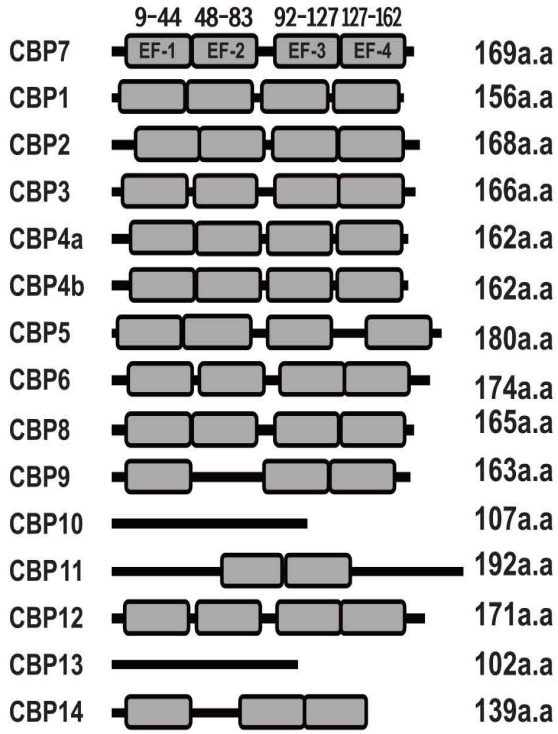

Fig. 1. Domain structure of CBP proteins in *Dictyostelium*. Domain structure of CBP7 showing four EF-hands.

3.2. Developmental defects induced by overexpression of CBP7

Upon starvation, *Dictyostelium* cells release cAMP, causing surrounding cells to migrate toward the cAMP source and initiate the multicellular developmental process[1]. During development, the extracellular Ca^{2+} level and the rate of Ca^{2+} influx are stimulated by chemoattractants in *Dictyostelium*[2]. To examine the possible roles of CBP7 in development, I performed a developmental assay (Fig. 2A). Wild-type cells and CBP7-RNAi cells

showed a normal developmental process, including the aggregation stage within 6 h, the slug stage within 12 h, and completion of fruiting body formation within 24 h. In contrast, Myc-CBP7 cells and GFP-CBP7 cells completely lost developmental ability, even no aggregation (Fig. 2A). Wild-type cells and CBP7-RNAi cells showed a normal developmental process, including the aggregation stage within 6 h, the slug stage within 12 h, and completion of fruiting body formation within 24 h. In contrast, Myc-CBP7 cells and GFP-CBP7 cells completely lost developmental ability, even no aggregation (Fig. 2A). High magnification observation displayed that wild-type cells and CBP7-RNAi cells aggregated within 6 h and formed fruit bodies within 24 h, but Myc-CBP7/GFP-CBP7 cells did not initiate the developmental process (Fig. 2B). These results showed that overexpression of CBP7 caused developmental defects.

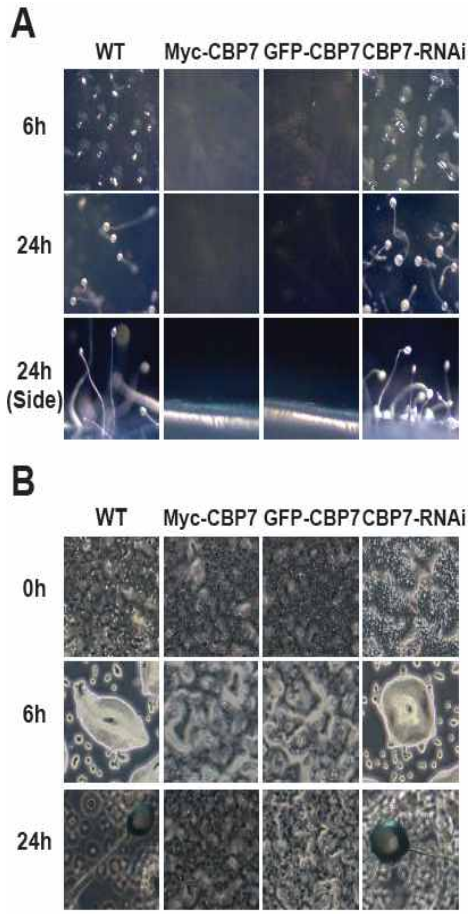

Fig. 2. Development of CBP7-overexpressing cells. (A) Developmental morphology of wild-type cells, Myc-CBP7 cells, GFP-CBP7 cells, and CBP7-RNAi cells. Vegetative cells were washed and plated on non-nutrient agar plates. Photographs were taken at the indicated times after plating. Developmental images of the cells at 6 h (Wild-type aggregation stage), 24 h (wild-type fruiting body stage) are shown. (B) High magnification of the developmental cells.

Table. 2. Overview of calcium binding proteins in *Dictyostelium*

Protein	Protein size	Location	EF-hand Domains	Molecular function	Disruption	Expressed
CBP1	166 a.a	Chromosome 2	4	Calcium ion binding	Regulates reorganization of the actin cytoskeleton	Expressed during the multicellular stages of development
CBP2	168 a.a	Chromosome 1	4	Calcium ion binding	-	More abundant in developing cells
CBP3	166 a.a	Chromosome 4	4	Calcium ion binding, actin binding	Located in the cell cortex	Expressed maximally at 6 hours of development
CBP4a	162 a.a	Chromosome 4	4	Calcium ion binding	Interacts with numA/nucleomorphin	Expressed in post-aggregation stage of development
CBP4b	162 a.a	Chromosome 4	4	Calcium ion binding	Interacts with numA/nucleomorphin	Expressed in post-aggregation stage of development
CBP5	180 a.a	Chromosome 2	4	Calcium ion binding	-	Expression peaks at the aggregation and slug stages
CBP6	174 a.a	Chromosome 2	4	Calcium ion binding	-	Expression peaks at the slug stage
CBP7	169 a.a	Chromosome 4	4	Calcium ion binding	-	Expression prior to the late culmination stage
CBP8	166 a.a	Chromosome 5	4	Calcium ion binding	-	Expressed at both slug and early culmination stages
CBP9	163 a.a	Chromosome 2	3	Metal ion binding	-	Expressed in porespore cells
CBP10	107 a.a	Chromosome 2	-	-	-	-
CBP11	192 a.a	Chromosome 4	4	Calcium ion binding	-	-
CBP12	171 a.a	Chromosome 4	4	Calcium ion binding	-	-
CBP13	102 a.a	Chromosome 4	-	-	-	-
CBP14	139 a.a	Chromosome 4	3	Calcium ion binding	-	-

3.3. Development at various calcium concentrations

CBP7-overexpressing cells did not develop on Na/K phosphate plates (Fig. 2). It seemed that cell aggregation was suppressed by low free calcium levels. To investigate whether development of CBP7-overexpressing cells is affected by extracellular calcium, I experimented with wild-type cells and GFP-CBP7 cells using different calcium concentrations in plates. The wild type exhibited a general aggregation step at 0 mM and 0.2 mM $CaCl_2$ concentrations within 6 h and formed a fruiting body within 24 h. It appeared that the development was suppressed at high calcium concentrations of 30 and 50 mM $CaCl_2$ (Fig. 3A). These results indicate that high calcium concentrations inhibit the development I observed the developmental morphology of GFP-CBP7 cells at 0, 0.2 (control), 30, and 50 mM $CaCl_2$ concentrations, but GFP-CBP7 cells did not develop on these plates (Fig. 3B). In addition, I examined the effect of extracellular calcium removal by EDTA chelation during development. High concentrations of EDTA removed calcium, and this inhibited development. As shown in Fig. 3A, wild-type cells were plated on Na/K plates containing 0, 2, 4, and 6 mM EDTA. In presence of 2 mM EDTA, the aggregation of wild-type cells was delayed at 6 h, and the formation of a fruiting body was delayed at 24 h. Development of wild-type cells was inhibited by high concentrations of EDTA (4 and 6 mM) (Fig. 4A). CBP7-overexpressing

cells did not develop on Na/K plates containing 0, 2, 4, and 6 mM EDTA (Fig. 4). The developmental phenotypes of CBP7-overexpressing cells were the same as those of wild-type cells developing on plates with high EDTA concentrations. These results indicate that the developmental process of CBP7-overexpressing cells is unaffected by the extracellular calcium concentration.

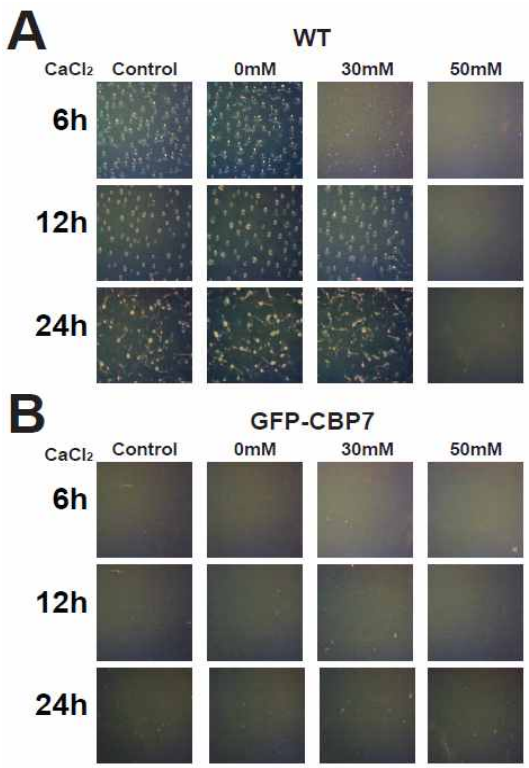

Fig. 3. Developmental phenotypes of the cells at various calcium concentrations. (A) Development of Wild-type cells on 0, 0.2 (control), 30, 50mM $CaCl_2$ concentration. (B) Development of GFP-CBP7 cells on 0, 0.2 (control), 30, 50 mM $CaCl_2$ concentration. Vegetative cells were washed and plated on Calcium plate. Photographs were taken at the time indicated after plating. Development at 6 h (Wild-type aggregation stage), 12 h (Wild-type slug stage), and 24 h (Wild-type fruiting body stage).

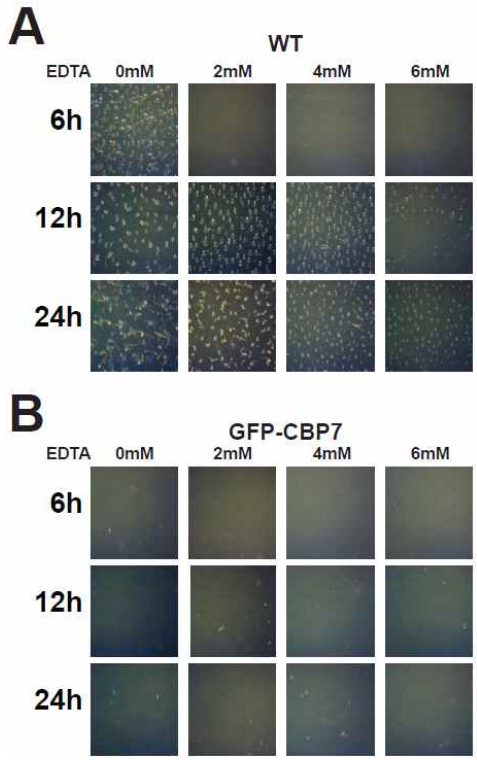

Fig. 4. Developmental phenotypes of the cells in the presence of EDTA. (A) Development of wild-type cells on 0 (Control), 2, 4, and 6 mM EDTA concentrations. (B) Development of GFP-CBP7 cells on 0 (Control), 2, 4, and 6mM EDTA concentrations. Vegetative cells were washed and plated on EDTA plate. Photographs were taken at the time indicated after plating. Development at 6 h (aggregation stage), 12 h (Wild-type tip forming stage), and 24 h (Wild-type fruiting body stage).

3.4. EF-hands in CBP7 are important in the process of development

Previous sequence analysis showed homology of CBP7 to other CBP proteins[5]. The EF-hands of CBP proteins have a helix-loop-helix

structure classified into two main sub-structures, a 12-residue loop and 14-residue EF-hands. These highly conserved helix-loop-helix structures and EF-hand motifs of CBP proteins usually bind Ca^{2+} ions[6]. Upon binding calcium, CBP proteins, such as CBP3, undergo conformational changes resulting in the exposure of hydrophobic residues. In the presence of calcium, CBP3 interacts with other proteins; its N-terminal domain in CBP3 has a role in sensing Ca^{2+}, and the C-terminal domain undergoes a conformational change and exposes its hydrophobic region[7]. CBP7 has four putative EF-hand motifs (residues D22-E33, D70-E81, D105-E116, and D140-E151) (Fig. 1). To investigate possible roles of the EF-hands of CBP7 in development, I performed point mutations into the EF-hands and prepared cells expressing point-mutated CBP7 proteins (Fig. 5A). All cells expressing the point-mutated CBP7 proteins showed normal development on Na/K plates. EF-hand 1 of CBP7 changes residues N24 and D26 to alanine; EF-hand 2 of CBP7 changes residues D63 and D65 to alanine; EF-hand 3 of CBP7 changes residues D107 and D109 to alanine; and EF-hand 4 of CBP7 changes residues D142 and D144 to alanine. Truncated EF-hands 3 and 4 of CBP7 change residues D107, D109, D142, and D144 to alanine, and truncated EF-hands 1 and 2 of CBP7 change residues D142 and D144 to alanine (Fig. 5). Cells with truncated EF-hands 3 and 4 of CBP7 showed normal aggregation and tip formation within 12 h, but development was slightly delayed, although fruiting bodies were finally formed (Fig. 6B). These results indicate that the EF-hand 3 and 4 domains play an important role in development.

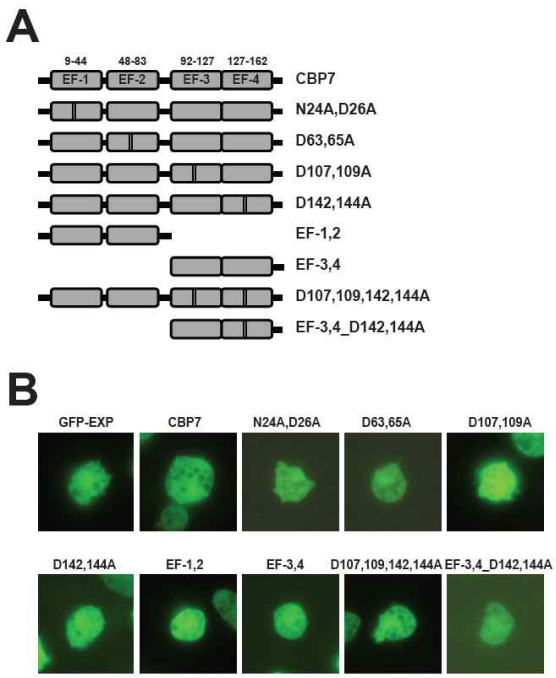

Fig. 5. Domain structure and localization of point-mutated CBP7 proteins. (A) Domain structure of the EF-hand point-mutated of CBP7 and truncated CBP7 proteins. (B) Localization of GFP-CBP7, CBP7N24, D26A, CBP7D63,65A CBP7D107,109A, CBP7D142,144A, CBP7D107,109,142,144A, △EF3,4, △EF1,2, △EF1,2D142,144A.

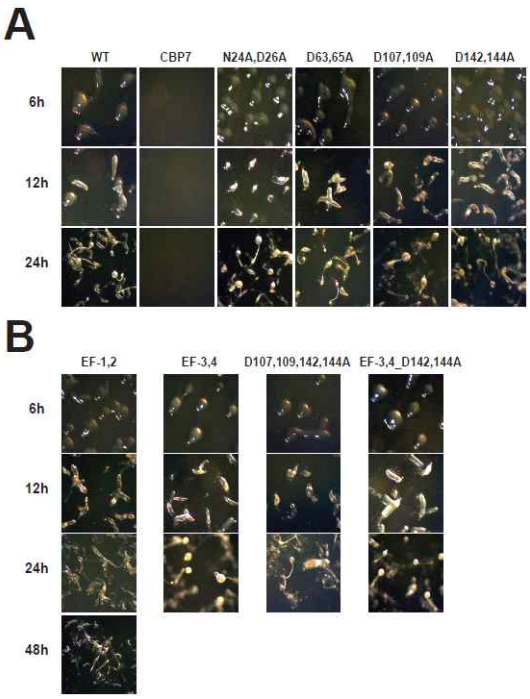

Fig. 6. Development of the cells expressing point-mutated CBP7 proteins. (A) Development of the cells expressing point-mutated CBP7 proteins. Wild-type, GFP-CBP7, CBP7N24, D26A, CBP7D63, 65A, CBP7D107, 109A, and CBP7D142, 144A. (B) Development of the cells expressing truncated CBP7 proteins. CBP7D107, 109,142,144A, △EF3,4, △EF1,2, and △EF1,2D142,144A. Vegetative cells were washed and plated on non-nutrient agar plates. Photographs were taken at the indicated times after plating. Developmental images of the cells at 6 h (Wild-type aggregation stage), 12 h (Wild-type tip forming stage), and 24 h (Wild-type fruiting body stage) are shown.

4. Discussion

The present study revealed the function of a novel CBP CBP7. CBP7 contains four EF-hand motifs and is homologous to the other 13 CBP proteins (Fig. 1).

CBP7 regulates developmental process

I investigated the specific functions of CBP7 in morphogenesis and development by overexpressing CBP7 or inhibiting the expression levels of CBP7. CBP1 and CBP 4 are expressed after the aggregation stage. CBP5 is expressed at the aggregation and slug stages in the developmental process. CBP6 is expressed at the slug stage. CBP7 is expressed at the late culmination stage in the pre-spore region. CBP8 is expressed at the slug stage. CBP3, CBP4b, CBP5, CBP6, CBP7, and CBP8 have been known as real calcium binding proteins[5]. CBP1 has been known to help reorganize the actin cytoskeleton in response to cAMP-induced changes in intracellular Ca^{2+}, and overexpression of CBP1 delayed the aggregation[8]. CBP3 has been known to interact with actin and is involved in slug migration[9]. Moreover, CBP3 undergoes conformational changes and exposes the hydrophobic region, which results in interactions with binding partners[7]. CBP4a has been known to regulate the number of nuclei in *Dictyostelium*[10]. In *Dictyostelium*, overexpression of chromatin assembly factor 1 (CAF1) has a stimulatory effect on

differentiation, but a caf1-null mutant exhibited normal development[11]. It is interesting that CBP7-overexpressing cells showed somewhat opposite effects to those of CAF1-overexpressing cells. Cells with CBP7-RNAi exhibited normal development. However, Myc-CBP7 and GFP-CBP7 cells exhibited failed to undergo the developmental process (Fig. 2). These data suggest that CBP7 may involve a different mechanism compared to those of other CBP proteins in the developmental process.

Functions of CBP7 EF-hands

Functions of CBP7 EF-hands in the regulation of the developmental process were investigated in the present study. It has been reported that CBP proteins have highly conserved EF-hand domains for Ca^{2+} binding and that EF-hands are rich in negatively charged amino acids, such as glutamic and aspartic acids[6]. The EF-hand Ca^{2+}-binding motif plays an essential role in eukaryotic cellular signaling, resulting in conformational changes in structures that are closely related to physiological functions. CBP3 has two EF-hand motifs, of which the N-terminal domain has a role of sensing Ca^{2+} and the C-terminal domain may undergo a conformational change and expose hydrophobic regions, which interact with hydrophobic regions of binding partners.

CBP7-overexpressing cells showed developmental defects; however, point-mutagenesis CBP7cells without truncated EF-hands 1 and 2 showed normal development, while CBP7 cells with truncated EF-hands 3 and 4 showed a delay in the formation of fruiting bodies. These data suggest that the C-terminal domain of CBP7 may interact with its binding partner, such as CBP3, and regulate the developmental process.

5. Conclusion

CBP proteins are involved in developmental processes in *Dictyostelium*. These CBP proteins have highly conserved EF-hand motifs, with Ca^{2+} ion binding to the interhelical loop region. In particular, CBP7 overexpressing cells showed complete loss of developmental process. The experiment using point-mutated CBP7 proteins showed that all EF-hand domains of CBP7 were important for CBP7 to function in the developmental process.

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