



CBP7 Interferes with the Multicellular Development of *Dictyostelium* Cells by Inhibiting Chemoattractant-Mediated Cell Aggregation

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Calcium ions are involved in the regulation of diverse cellular processes. Fourteen genes encoding calcium binding proteins have been identified in *Dictyostelium*. CBP7, one of the 14 CBPs, is composed of 169 amino acids and contains four EF-hand motifs. Here, we investigated the roles of CBP7 in the development and cell migration of *Dictyostelium* cells and found that high levels of CBP7 exerted a negative effect on cells aggregation during development, possibly by inhibiting chemoattractant-directed cell migration. While cells lacking CBP7 exhibited normal development and chemotaxis similar that of wild-type cells, CBP7 overexpressing cells completely lost their chemotactic abilities to move toward increasing cAMP concentrations. This resulted in inhibition of cellular aggregation, a process required for forming multicellular organisms during development. Low levels of cytosolic free calcium were observed in CBP7 overexpressing cells, which was likely the underlying cause of their lack of chemotaxis. Our results demonstrate that CBP7 plays an important role in cell spreading and cell-substrate adhesion. *cbp7* null cells showed decreased cell size and cell-substrate adhesion. The present study contributes to further understanding the role of calcium signaling in regulation of cell migration and development.

Keywords: calcium binding proteins, cell migration, development, dictyostelium

INTRODUCTION

Calcium ions are involved in the regulation of diverse cellular processes such as chemotaxis, cell adhesion, and multicellular development (Clapham, 2007; Lusche et al., 2009; Siu et al., 2011). Calcium ions regulate cellular processes through their interactions with calcium-binding proteins (CBPs). Calcium-binding proteins function as calcium buffers to control the intracellular concentration of calcium ions or as calcium sensors to transduce signals to a series of downstream effectors (Chin and Means, 2000; Clapham, 2007).

Dictyostelium discoideum is a unicellular eukaryotic microorganism used as a model system to address many important cellular processes including cell migration, cell division, phagocytosis, and development (Chisholm and Firtel, 2004; Lee and Jeon, 2012; Siu et al., 2011). Upon starvation, *Dictyostelium* initiates a multicellular developmental process by forming aggregates, slugs, and finally, fruiting bodies. In the initial stages of this developmental process, *Dictyostelium* cells emit the chemoattractant, cAMP, which cause cells to migrate in the direction of increasing concentrations along the gradient to form aggregates (Chisholm and Firtel, 2004). It has been shown that the rate of Ca^{2+} influx was stimulated by the chemoattractant, cAMP, and that the intracellular calcium ions affected cell-cell adhesion and cell fate determination (Chisholm and Firtel, 2004; Malchow et al., 1996; Yumura et al., 1996).

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Fourteen calcium-binding proteins (CBP) have been identified in *Dictyostelium*. The expressions of these proteins are tightly linked to their multicellular stages of development. CBP1 is expressed prior to cell aggregation and associates with the actin cytoskeleton. CBP1 is suggested to regulate the reorganization of the actin cytoskeleton during cell aggregation (Dharamsi et al., 2000; Dorywalska et al., 2000; Sakamoto et al., 2003). *cbp1* null cells showed delayed aggregation and development (Dharamsi et al., 2000). CBP1 also interacts with another calcium-binding protein, CBP4a, and the actin-binding proteins, protovillin and EF-1a, in yeast two-hybrid experiments (Dorywalska et al., 2000). The function of CBP2 is unknown, but its mRNA concentrations was shown to peak during cellular aggregation and then decrease after 12 h, suggesting that it specifically functions during distinct stages of development (Andre et al., 1996). CBP3 is relatively well studied, and actin 8 was identified as an interacting protein with CBP3 in yeast two-hybrid screening. Cells overexpressing CBP3 showed accelerated cell aggregation and increased number of small aggregates and fruiting body. It was suggested that CBP3 interacts with the actin cytoskeleton and plays important roles in cell aggregation and slug migration during development (Lee et al., 2005; Mishig-Ochiriin et al., 2005). CBP4a is a nucleolar protein that interacts with nucleomorphin, which is a cell cycle checkpoint protein, in Ca²⁺-dependent manner. CBP4a was suggested to function during mitosis (Catalano and O'Day, 2013; Myre and O'Day, 2004). CBP5, 6, 7, and 8 contain canonical EF-hand motifs, which mediate their Ca²⁺-binding properties. These proteins are under spatial and temporal regulation during development and might have specific roles in cellular processes such as cell migration, cell adhesion, and development (Sakamoto et al., 2003). However, the exact functions of these proteins remain unknown. Here, we investigated the functions of CBP7, one of the CBP proteins, in cell migration and development by examining the characteristics of cells lacking or overexpressing CBP7.

MATERIALS AND METHODS

Strains and plasmid construction

Dictyostelium wild-type KAx-3 cells were cultured axenically in HL5 medium or in association with *Klebsiella aerogenes* at 22°C. The knock-out strains and transformants were maintained in 10 µg/ml blasticidin or 10 µg/ml of G418. The full coding sequence of *cbp7* cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) and cloned into the *EcoRI* - *XhoI* site of the expression vector pEXP-4(+) containing a GFP or Myc fragment. The plasmids were transformed into KAx-3 cells or *cbp7* null cells. The *cbp7* knock-out construct was made by inserting the blasticidin resistance cassette (*bsr*) into *BglII* site created at nucleotide 415 of *cbp7* gDNA and used for a gene replacement in KAx-3 parental strains. Randomly selected clones were screened for a gene disruption by PCR. The primers used in the screening for a gene replacement are following; a forward primer I (5'-GAATTCATGAGCACTGTGGTGATAATAG-3') and reverse primers II (5'-CTCGATAGTCTCAGCATTTTGTCAATTTG-3'), III (5'-CTCGATTAAACAAATTGGACCTCTTGC-3'), and IV

(5'-GATTAATGTGGTATTTTGTCCCAAGAG-3').

Cell adhesion assay

Cell adhesion assay was performed as described previously (Mun et al., 2014). Log-phase growing cells on the plates were washed and resuspended at a density of 2×10^6 cells/ml in 12 mM Na/K phosphate buffer. 200 µl of the cells were placed and attached on the 6-well culture dishes. Before shaking the plates, the cells were photographed and counted for calculating the total cell number. To detach the cells from the plates, the plates were constantly shaken at 150 rpm for 1 h, and then the attached cells were photographed and counted (attached cells) after the medium containing the detached cells was removed. Cell adhesion was presented as a percentage of attached cells compared with total cells.

Development

Development was performed as described previously (Jeon et al., 2009). Exponentially growing cells were harvested and washed twice with 12 mM Na/K phosphate buffer (pH 6.1) and resuspended at a density of 3.5×10^7 cells/ml. 50 µl of the cells were placed on Na/K phosphate agar plates and developed for 24 h. For development of the cells under submerged conditions, exponentially growing cells (2×10^6 cells) were placed and developed in 12-well plates containing Na/K phosphate buffer. The multicellular developmental organisms was photographed and examined with a phase-contrast microscope at the indicated times in the figures.

Chemotaxis

Chemotaxis towards cAMP was examined as described previously (Jeon et al., 2007b; Mun et al., 2014). The aggregation-competent cells were prepared by incubating the cells at a density of 5×10^6 cells/ml in Na/K phosphate buffer for 10 h. Cell migration was analyzed using a Dunn Chemotaxis Chamber (Hawksley). The images of chemotaxing cells were taken at time-lapse intervals of 6 s for 30 min using an inverted microscope (IX71; Olympus). The data were analyzed using the NIS-Elements software (Nikon) and Image J software (National Institutes of Health). For examining cell migration in the aggregation stage of development, 2.5% of RFP-labeled wild-type cells and 2.5% of cells expressing GFP-CBP7 were mixed with 95% of unlabeled wild-type cells and developed on Na/K phosphate agar plates. The fluorescence images of moving cells at the aggregation step of development were captured by the NIS-Elements software, and the movement of fluorescent cells was traced and analyzed using the Image J software. 'Trajectory speed' was used to quantify motility of the cells. The trajectory speed is the total distance travelled of a cell divided by time. 'Directionality' is a measure of how straight the cells move. Cells moving in a straight line have a directionality of 1.0. It was calculated as the distance moved over the linear distance between the start and the finish.

Measurement of cytosolic calcium

Fluo-4 AM, a fluo calcium indicator, was obtained from Molecular Probes, and the cells were labeled with fluo-4 AM

according to the protocol provided by manufactures. *Dictyostelium* cells at a density of 5×10^6 cells/ml in 1 x PBS buffer were added by 2.2 mM Fluo-4 AM (final concentration, 8 μ M). The mixed solutions were transferred into 96-well plates and incubated in the dark at room temperature for 60 min, followed by washing gently twice with 1 x PBS buffer. The fluorescence levels of the cells in the wells were quantified using a fluorescence microplate reader (Molecular Devices) and SoftMax Pro software. The excitation and emission wavelengths were 494 nm and 506 nm, respectively.

RT-PCR

The total RNAs from wild-type cells and *cbp7* null cells were extracted by using the SV Total RNA Isolation system (Promega), and the cDNAs were synthesized by reverse transcription with MMLV reverse transcriptase (Promega) using random hexamers and 5 μ g of total RNAs. 5 μ l of the cDNAs were used in the following PCR with 35 cycles employing gene-specific primers. The universal 18S ribosomal RNA specific primers were used as an internal control (Jeon et al., 2007a).

Statistical analysis

The results were expressed as the mean \pm standard deviation (SD) (at least three independent experiments). Data were analyzed using Student's two-tailed *t* test. $*p < 0.05$ was considered to be statistically significant.

RESULTS

CBP7, a calcium-binding protein

There are 14 genes encoding CBP proteins in the genomes of *Dictyostelium*. The putative domain structures are depicted (Supplementary Fig. S1). Most of the CBP proteins (CBP 1-8 and CBP12) have similar numbers of residues and 4 EF-hand motifs. CBP9 and 14 have three EF-hand motifs. Among them, only CBP1, CBP2, CBP3, CBP4a, and CBP4b

have been previously characterized. Here, we investigated one of the 14 CBP proteins, CBP7. *Dictyostelium* CBP7 has 169 amino acids (expected molecular mass of 19.3 kDa) and 4 EF-hand motifs (Supplementary Fig. S1A). The phylogenetic trees of the CBP proteins containing four EF-hand motifs illustrate that CBP7 is closely related to the CBP3, 6, and 12 (Supplementary Fig. S1C). A multiple alignment of CBP7 with other CBP proteins shows that CBP7 has 74%, 70%, and 68% amino acid identities with CBP6, CBP12, and CBP3, respectively, and contains the conserved residues in all 4 EF-hands that are necessary for calcium binding (Supplementary Fig. S2). CBP7 is known as a real Ca^{2+} -binding protein (Sakamoto et al., 2003).

To investigate the functions of CBP7, we prepared *cbp7* knock-out strains by homologous recombination with the *cbp7* knock-out DNA construct containing a blasticidin resistance (*bsr*) antibiotic cassette into the *cbp7* genomic DNA of KAX-3 parental strains (Supplementary Fig. S3). *cbp7* knock-out cells were confirmed by polymerase chain reactions (PCR) (Supplementary Fig. S3). PCR with a set of primers, I/II and I/IV, produced bands of 361 and 826 bp in wild-type cells and bands of 361 and 2176 bp in *cbp7* null cells, respectively. The increased size (2176 bp in *cbp7* null cells) was consistent with the insertion of the *bsr* cassette into the gene (Supplementary Fig. S3). Reverse transcription (RT)-PCR using the primer set I/III and cDNA from wild-type and *cbp7* null cells confirmed that the *cbp7* gene was not transcribed in the *cbp7* null cells. No band was detected in RT-PCR experiments using cDNA from *cbp7* null cells (lane 1), while a band of 510 bp was observed in RT-PCR experiments using cDNA from wild-type cells (lane 2). To examine the functions of CBP7, cells overexpressing GFP-CBP7 fusion proteins (expected molecular mass of 46 kDa) were prepared, and the expression of the protein was confirmed by western blotting using anti-GFP antibodies (Supplementary Fig. S3). GFP-CBP7 was observed in the cytosol of cells (data not shown).

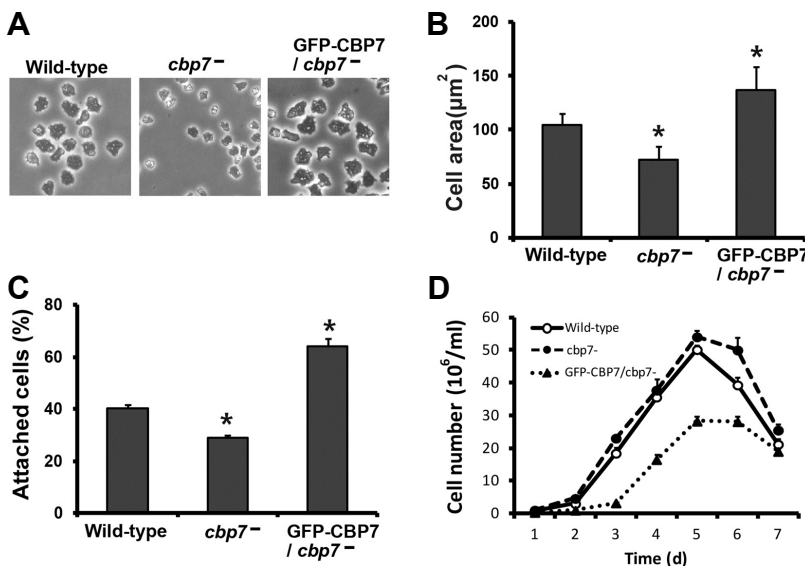


Fig. 1. Cell spreading, cell adhesion, and growth rate of the cells. (A) Morphology of wild-type cells, *cbp7* null cells, and *cbp7* null cells expressing GFP-CBP7. Exponentially growing cells were photographed. (B) Measurement of cell area. The area of the cells was measured using ImageJ software. The values are the means \pm SD of three independent experiments ($*p < 0.05$ compared to the control by the student's *t*-test). (C) Cell-substrate adhesion. Adhesion of the cells to the substrate was expressed as a percentage of attached cells to total cells ($*p < 0.05$ compared to the control). (D) Growth rates of the cells. Wild-type cells, *cbp7* null cells, and *cbp7* null cells expressing GFP-CBP7 were cultured with a constant shaking of 150 rpm and counted at intervals thereafter. The means \pm SD were plotted from three independent experiments.

CBP7 is involved in the control of cell morphology and cell adhesion

We first examined the morphology of *cbp7* null cells and GFP-CBP7 overexpressing cells (GFP-CBP7 cells) (Fig. 1). *cbp7* null cells were smaller and more rounded than wild-type cells. In contrast, GFP-CBP7 cells were more spread and flattened than wild-type cells and *cbp7* null cells. Measurement of cell areas using the NIS-Element software showed that *cbp7* null cells were approximately half the size of wild-type cells, and GFP-CBP7 cells were 1.4-fold larger than wild-type cells (Figs. 1A and 1B). Next, we investigated cell adhesion of the cells by measuring the fraction of cells that attached to the plate during agitation. Compared to wild-type cells, cells lacking CBP7 showed decreased cell adhesion (Fig. 1C). GFP-CBP7 cells exhibited highly increased cell-substrate adhesion (Fig. 1C). The growth rates of *cbp7* null cells were similar to those of wild-type cells. However, GFP-CBP7 cells showed slower growth rates compared to both the *cbp7* null and wild-type cells (Fig. 1D). These results indicate that CBP7 is required for cell spreading and cell-substrate adhesion.

Overexpression of CBP7 resulted in inhibition of development

Upon starvation, *Dictyostelium* cells release cAMP, causing surrounding cells to migrate toward the cAMP source and initiate their multicellular developmental process (Chisholm and Firtel, 2004). During development, the influx of the extracellular Ca^{2+} is stimulated by chemoattractants in *Dictyostelium* (Tanaka et al., 1998). To investigate the possible roles of CBP7 in development, we examined the developmental processes of the cells (Fig. 2). Wild-type cells and *cbp7* null cells exhibited a normal developmental process, with the aggregation stage occurring within 6 h, the slug stage within 12 h, and formation of fruiting bodies within 24 h. In contrast, GFP-CBP7 cells completely lost developmental ability, even aggregation (Fig. 2A). Wild-type cells expressing GFP-CBP7 or Myc-CBP7 failed to develop, which was similar to the observation in *cbp7* null cells expressing GFP-CBP7 (Data not shown).

To further investigate impairment of the aggregation stage in GFP-CBP7 cells, we examined the aggregation abilities of cells by placing them on 12-well plates containing developmental buffers instead of agar plates (Fig. 2B). In developmental buffer, wild-type cells and *cbp7* null cells started to aggregate towards an aggregation center within 6 h and formed small tight aggregates within 10 h (Fig. 2B). Contrary to wild-type cells and *cbp7* null cells, GFP-CBP7 cells did not aggregate. These results indicate that overexpression of CBP7 results in severe defects in aggregation and suggest that CBP7 is dispensable to the multicellular developmental process of *Dictyostelium* cells but plays an important inhibitory role in the initial aggregation stage of development.

Overexpression of CBP7 resulted in loss of directional cell migration

In contrast with wild-type and *cbp7* null cells, GFP-CBP7 cells showed no aggregation when deprived of nutrients (Fig. 2). These data suggest that GFP-CBP7 cells may have defects in

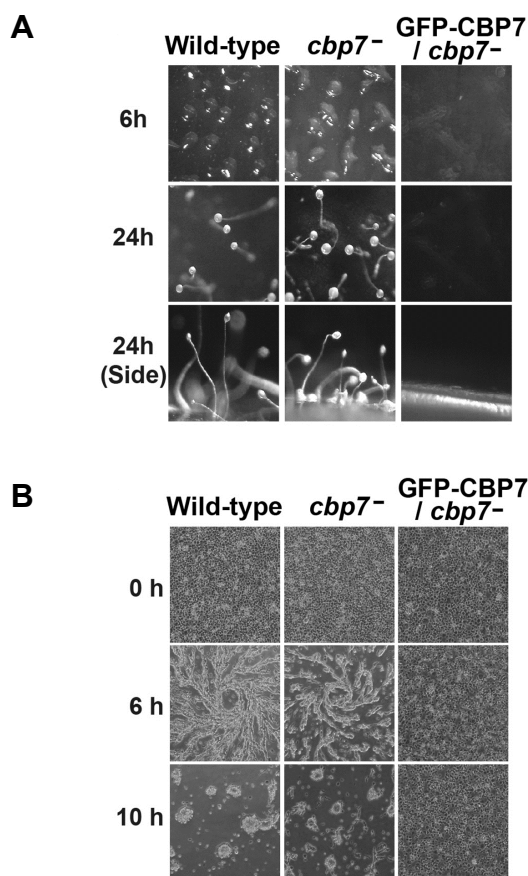


Fig. 2. Development of wild-type cells, *cbp7* null cells, and GFP-CBP7 overexpressing cells. (A) Development on non-nutrient agar plate. Exponentially growing cells were washed and plated on non-nutrient agar plates. Photographs were taken at the indicated times after plating. Representative developmental images of the cells at 6 h (aggregation stage) and at 24 h (fruiting body formation stage) are shown. Side views of fruiting bodies at 24 h are shown at the bottom row. (B) Development under submerged conditions. The cells were grown in 12-well plates. Development of the cells was induced by changing the media with non-nutrient Na/K phosphate buffer. Photographs were taken at the indicated times after induction of development.

chemoattractant-directed cell migration, which occurs in the initial aggregation stage of cellular development. To test this hypothesis, we performed cAMP-directed cell migration experiments using a Dunn chemotaxis chamber (Fig. 3). Aggregation-competent cells were prepared by starving the cells in Na/K phosphate buffer for 10 h (Mun et al., 2014). Wild-type cells had high moving speeds (9.1 $\mu\text{m}/\text{min}$) and directionality (0.9), which is a measure of how straight the cells move toward the chemoattractant. *cbp7* null cells moved toward increasing cAMP concentrations with similar moving speeds (10.8 $\mu\text{m}/\text{min}$) and directionality (0.8) to those of wild-type cells. In contrast, GFP-CBP7 cells had significantly decreased migration speeds (5.5 $\mu\text{m}/\text{min}$) and directionality

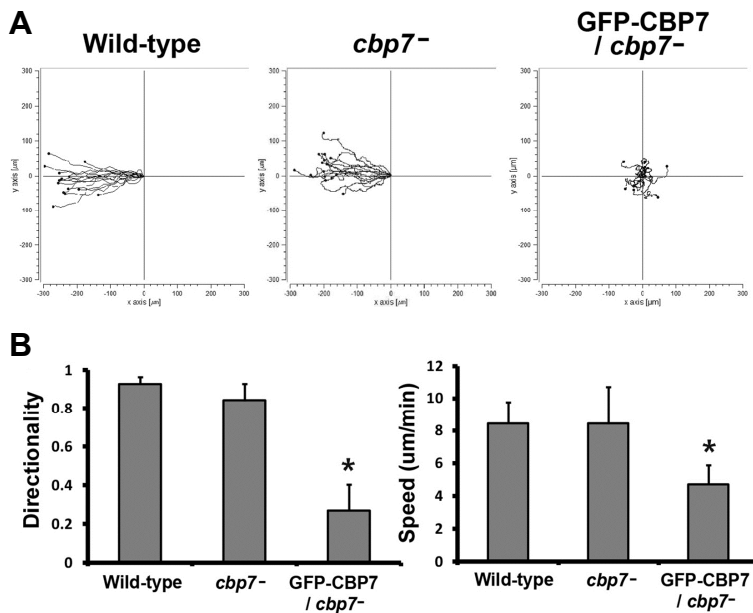


Fig. 3. Chemotaxis of wild-type cells, *cbp7* null cells, and CBP7 overexpressing cells. Aggregation-competent cells were placed in a Dunn chemotaxis chamber, and the movements of the cells up a chemoattractant, cAMP, gradient were recorded by time lapse photography for 30 min at 6 s intervals. (A) Trajectories of cells migrating toward cAMP in a Dunn chemotaxis chamber. Plots show migration paths of the cells with the start position of each cell centered at point 0.0. Cells migrate toward the increasing gradients of cAMP on the left. Each line represents the track of a single cell chemotaxing toward cAMP (150 μM). (B) Analysis of chemotaxing cells. The recorded images were analyzed by ImageJ software. Directionality is a measure of how straight the cells move. Cells moving in a straight line have a directionality of 1.0. Speed indicates the speed of the cells movements along the total path. Error bars represent SD. Statistically different from control at * $p < 0.05$ by the student's *t*-test.

(0.26) compared to wild-type cells and *cbp7* null cells. GFP-CBP7 cells appeared to lose directionality and move randomly within the cAMP gradient (Fig. 3). These results suggest that CBP7 may negatively impact cell aggregation by inhibiting cAMP-mediated directional cell migration in the aggregation stage of development.

These results related to cAMP-dependent chemotaxis were further confirmed using a cell migration assay with chimeric cells containing 95% unlabeled wild-type cells, 2.5% RFP-labeled wild-type cells, and 2.5% GFP-CBP7 expressing cells. All cells were simultaneously starved of nutrients, and the migration speeds of the labeled cells were

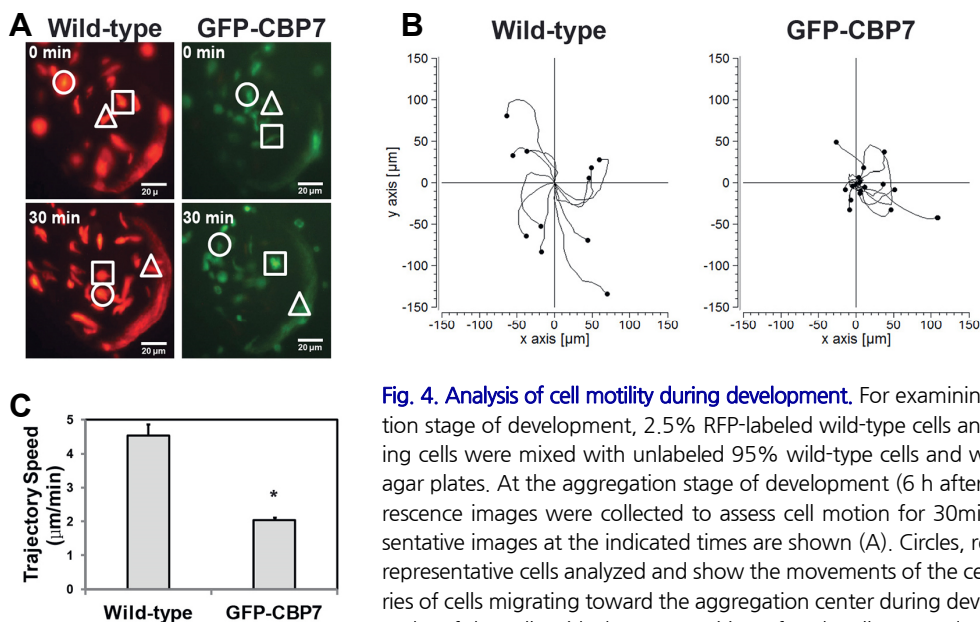


Fig. 4. Analysis of cell motility during development. For examining cell migration in the aggregation stage of development, 2.5% RFP-labeled wild-type cells and 2.5% GFP-CBP7 overexpressing cells were mixed with unlabeled 95% wild-type cells and were developed on non-nutrient agar plates. At the aggregation stage of development (6 h after development), time-lapse fluorescence images were collected to assess cell motion for 30 min at 1 min intervals. Two representative images at the indicated times are shown (A). Circles, rectangles, and triangles indicate representative cells analyzed and show the movements of the cells for aggregation. (B) Trajectories of cells migrating toward the aggregation center during development. Plots show migration paths of the cells with the start position of each cell centered at point 0.0. Each line represents the track of a single cell migrating toward the center of a circular aggregate. (C) Trajectory speeds of wild-type cells and GFP-CBP7 cells. The recorded images were analyzed by ImageJ software. Speed indicates the speed of the cells movements along the total path. Error bars represent SD. Statistically different from control at * $p < 0.05$ by the student's *t*-test.

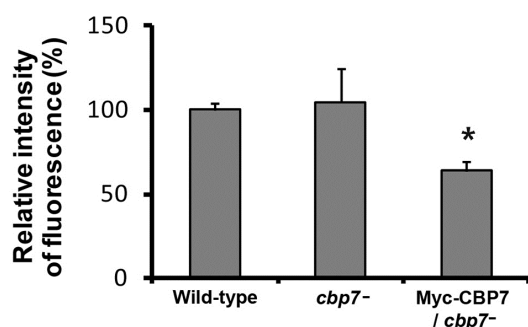


Fig. 5. Measurement of the cytosolic calcium levels. Cytosolic calcium levels in the cells were determined using the fluo-4 AM calcium indicator and a fluorescence microplate reader (excitation/emission wavelengths, 494/516 nm). Error bars represent SD. Statistically different from control at * $p < 0.05$ by the student's t -test.

measured during the aggregation stage of development (Fig. 4). Wild-type cells exhibited a moderate moving speed (4.53 $\mu\text{m}/\text{min}$) that was significantly higher than GFP-CBP7 cells (2.03 $\mu\text{m}/\text{min}$) during aggregation at the 6 h time point (Fig. 4), indicating that GFP-CBP7 cells have a defect in forming aggregates by cAMP-dependent chemotaxis to the center of aggregation.

Overexpression of CBP7 decreased the cytosolic calcium concentration

Influx of Ca^{2+} and elevation of its level by cAMP are important in aggregation of *Dictyostelium* cells during development (Chisholm and Firtel, 2004; Siu et al., 2011). The calcium concentration in *Dictyostelium* rapidly increases when cell aggregation occurs (Tanaka et al., 1998). Since CBP7 is known as a real calcium-binding protein (Sakamoto et al., 2003), it was postulated that the cytosolic calcium concentration might be altered by the presence of CBP7. We determined the cytosolic calcium concentration in cells by measuring the intensity of fluorescence of the Fluo-4 calcium indicator using a fluorescence microplate reader. *cbp7* null cells had slightly higher concentrations of cytosolic calcium compared to wild-type cells. More interestingly, *cbp7* null cells expressing Myc-CBP7 exhibited significantly lower levels of free intracellular calcium than wild-type cells and *cbp7* null cells (Fig. 5). These results suggest that deregulation of free intracellular calcium levels may result in developmental defects in CBP7 overexpressing cells.

DISCUSSION

Our results demonstrate that CBP7 is required for cell spreading and cell-substrate adhesion and has a negative impact on multicellular development, possibly through inhibition of chemoattractant-mediated cell migration in the initial aggregation stage of development. *cbp7* null cells showed decreased cell size and cell-substrate adhesion. GFP-CBP7 overexpressing cells (GFP-CBP7 cells) had significantly increased cell size and cell-substrate adhesion compared to

wild-type cells. Unexpectedly, overexpression of CBP7 caused severe defects in development. When deprived of nutrients, CBP7 overexpressing cells completely lost their chemotactic abilities to move toward increasing concentrations of the chemoattractant, cAMP, resulting in no cellular aggregation and formation of multicellular organisms. Cells lacking CBP7 showed normal cell migration and developed as the wild-type cells. These results suggest that CBP7 is not required for cell migration or development and that CBP7 functions as an inhibitor of cell aggregation by disrupting chemotaxis during development. Thus, during normal development of *Dictyostelium* cells, our results suggest that CBP7 should be maintained at low levels at the aggregation stage of development. In support of this conclusion, it has been reported by dictyExpress and Sakamoto et al. (2003) that CBP7 is not expressed in the vegetative state, is expressed during the slug stage of development, and then disappears at the late culmination stage (Rot et al., 2009; Sakamoto et al., 2003).

We propose one possible mechanism by which CBP7 may inhibit cell migration and development, a hypothesis that should be addressed in future studies. Low levels of intracellular calcium in CBP7 overexpressing cells resulted in the loss of directional cell migration during the aggregation stage of development. CBP7 has four highly conserved EF-hand motifs for Ca^{2+} binding, which are rich in negatively charged amino acids such as glutamic acids and aspartic acids (Gifford et al., 2007) and has been demonstrated as a calcium binding protein through Ca^{2+} -overlay experiments (Sakamoto et al., 2003). In this study, measurement of the free intracellular calcium levels revealed that overexpression of CBP7 resulted in significantly lower levels of calcium in the cytosol and that loss of CBP7 caused slightly increased levels of intracellular calcium compared to wild-type cells. Based on previously reported data and the results presented herein, CBP7 proteins appear to directly bind to free intracellular calcium and function as a calcium buffer to lower the levels of intracellular calcium. Low level of intracellular calcium in CBP7 overexpressing cells might affect chemoattractant-directed cell migration. In agreement with our results, many studies have demonstrated that calcium ions are involved in cell migration. In *Dictyostelium* cells, Ca^{2+} -influx is stimulated by chemoattractants, which are emitted from the cells during development. Elevated intracellular Ca^{2+} level was reported to play a role in cell contraction, which is mediated by the actomyosin cytoskeleton (Malchow et al., 1996; Tanaka et al., 1998; Yumura et al., 1996). In macrophages, it was reported that Ca^{2+} influx was required for positive feedback at the leading edge of polarized cells. Inhibition of extracellular Ca^{2+} influx leads to a loss of differential leading-edge activation of PI3K and F-actin assembly (Evans and Falke, 2007).

Another possibility is that CBP7 is both a calcium sensor and a downstream effector of calcium ions, as was illustrated for CBP3 (Lee et al., 2005; Mishig-Ochiriin et al., 2005). CBP3 has been shown to interact with the actin cytoskeleton and play important roles in cell aggregation and slug migration during development (Lee et al., 2005). Moreover, CBP3 undergoes conformational changes upon binding to Ca^{2+} , which allows for interactions with binding partners (Mishig-

Ochiriin et al., 2005). However, the roles of CBP7 seem to be opposite to those of CBP3. It was reported that cells overexpressing CBP3 showed accelerated cellular aggregation and increased numbers of small aggregates and fruiting body (Lee et al., 2005), whereas CBP7 overexpressing cells displayed no cell aggregation and complete loss of development. A large number of proteins have been identified as CBP protein-binding partners. CBP1 and CBP3 interact with the actin cytoskeleton and CBP1 also interacts with another calcium-binding protein, CBP4a, and the actin-binding proteins, protovillin and EF-1a, in yeast two-hybrid experiments (Dharamsi et al., 2000; Dorywalska et al., 2000). Nucleomorphin, a cell cycle checkpoint protein, is a known binding protein of CBP4a (Catalano and O'Day, 2013; Myre and O'Day, 2004). Further experiments are in progress to determine CBP7-binding proteins.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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