creative commons
COMMONS DEED

저작자표시 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.
- 이차적 저작물을 작성할 수 있습니다.
- 이 저작물을 영리 목적으로 이용할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

조선대학교
CHOSUN UNIVERSITY

[UCI]I804:24011-200000265909

2017년 2월
석사학위논문

Regulation of Cellular Developmental Process by a Calcium Binding Protein CBP7 in *Dictyostelium*

조 선 대 학 교 대 학 원

생 명 과 학 과

신 동 엽

조선대학교
CHOSUN UNIVERSITY

Regulation of Cellular Developmental Process by a Calcium Binding Protein CBP7 in *Dictyostelium*

칼슘 결합 단백질 CBP7에 의한
딕티오스텔리움 세포 발생 과정 조절

2017 년 2 월 24 일

조 선 대 학 교 대 학 원

생 명 과 학 과

신 동 엽

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Regulation of Cellular Developmental Process by a Calcium Binding Protein CBP7 in *Dictyostelium*

지도교수 전택중

이 논문을 이학석사학위 신청 논문으로 제출함

2016년 10월

조 선 대 학 교 대 학 원

생 명 과 학 과

신 동 엽

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

신동엽의 석사학위논문을 인준함

위원장 조선대학교 교 수 조 광 원 (인)

위 원 조선대학교 교 수 송 상 기 (인)

위 원 조선대학교 교 수 전 택 중 (인)

2016년 11월

조 선 대 학 교 대 학 원

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

CONTENTS

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

LIST OF TABLES

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

LIST OF FIGURES

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
CBP	Calcium binding protein
EDTA	Ethylenediaminetetraacetic acid
Fluo4-AM	Non-fluorescent acetoxymethyl ester
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
iplA	Inositol 1,4,5-trisphosphate receptor-like protein
RA	Ras association
RBD	Ras-binding domain
RFP	Red fluorescent protein

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

ABSTRACT

Regulation of Cellular Developmental Process by a Calcium Binding Protein CBP7 in *Dictyostelium*

Dongyeop SHIN

Advisor: Associate Prof. Taeck Joong Jeon, Ph.D.

Department of Life Science,

Graduate School of Chosun University

Calcium ions play an important role in development and intracellular signaling. Fourteen different genes encoding small calcium-binding proteins (CBPs) have been identified in *Dictyostelium*. However, most of CBPs in the developmental process have not been studied yet. In this study, I investigated functions of CBP7, one of the 14 CBPs, in morphogenesis, adhesion, growth, and development by analyzing phenotypes of the cells expressing the CBP7 RNA interference constructs (CBP7-RNAi), the full-length wild-type CBP, or point-mutated CBP7 proteins. CBP7 is composed of 169 amino acids and contains four EF-hand domains. CBP7 showed significant sequence homology to CBP3, CBP6, and CBP12, which are involved in cell aggregation and adhesion. CBP7-

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

overexpressing cells showed increased cell size and strong adhesion compared with wild-type cells. In contrast, the growth rate of CBP7-overexpressing cells was lower than that of the wild-type cells. The CBP7-overexpressing cells showed complete loss of developmental process. These cells remained at 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. In addition, CBP7-overexpressing cells exhibited defects in chemotaxis and low level of cytosolic free calcium concentration. The experiments using point-mutated CBP7 proteins showed that all EF-hand domains of CBP7 were important for CBP7 to function in the developmental process. Yeast two-hybrid experiments using CBP7 as a bait showed that CBP7 specifically binds to RasG. These results suggest that CBP7 has important roles in the development process through all of its EF-hand domains and by binding to RasG. The present study would contribute to further understand the mechanism by which calcium regulates development and the relationship between the calcium signaling and Ras signaling pathways.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

국문초록

칼슘 결합 단백질 CBP7에 의한
딕티오스텔리움 세포 발생 과정 조절

칼슘 이온은 발달 및 세포 내 신호 전달에 중요한 역할을 한다. *Dictyostelium* 에서는 칼슘 결합 단백질 (CBPs)을 코딩 하는 14 가지 유전자가 있지만, 대부분의 CBP는 발생 과정에서 어떤 기능을 하는지 아직 연구되지 않았다. 본 연구에서는, CBP7의 RNA 간섭 세포주, CBP7 과발현 세포주, EF-hand 도메인의 점 돌연변이 세포주 및 절편 단백질 발현 세포주를 이용하여 세포의 형태형성, 부착, 성장, 발생에서 14개의 CBP 중 하나인 CBP7의 특이적인 기능들이 무엇인지를 밝히고자 하였다. CBP7은 169 개의 아미노산으로 구성되어 있으며 4 개의 EF-hand 도메인을 포함하고 있다. CBP7은 세포 응집에 관여하는 CBP3, CBP6 및 CBP12와 유의한 서열 상동성을 보였다. CBP7 과발현 세포는 야생형 세포에 비해 세포 크기가 증가하고 강한 부착을 보였다. 반면, CBP7 과발현 세포의 성장 속도는 야생형 세포의 성장 속도보다 낮았다. CBP7 과발현 세포는 발생 과정이 전혀 일어나지 않았다. 이 세포들은 발달 유도 조건 하에서 단일 세포 성장 단계에 머물러 있는 반면, 야생형 세포는 6-8 시간 내에 발달을 시작하여 과실체를 형성하였다. 또한, CBP7 과발현 세포는 주화성 세포이동에서 결함을 보였으며 낮은 수준의 세포질 내 자유 칼슘 농도가 야생종에 비해 낮았다. 점 돌연

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

변이를 시킨 CBP7단백질을 이용한 실험결과, CBP7의 모든 EF-hand domain이 CBP7이 발달 과정에서 기능하는 데 중요하다는 것을 보여 주었다. CBP7을 미끼로 사용하는 yeast two-hybrid 실험결과 CBP7이 RasG와 특이적으로 결합한다는 것을 보여 주었다. 이러한 결과는 CBP7이 모든 EF-hand 도메인을 통한 발달 과정과 RasG에 결합 함으로써 중요한 역할을 한다는 것을 시사한다. 칼슘에 의한 발생과정 조절 기전과 칼슘/라스 신호전달 경로의 연관성을 이해하는데 기여할 것이다.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

I. INTRODUCTION

Calcium ions are required for the regulation of a variety of cellular processes and intracellular signaling, such as the chemotaxis, development, and differentiation (Marks and Maxfield, 1990; Van Haastert, 1995). 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 (Chin and Means, 2000).

Cell migration is involved in various biological processes, including tumor metastasis, embryonic development, and wound healing (Lee and Jeon, 2012). Cell migration requires continuous, coordinated cell attachment and detachment. The cell migration mechanism involves the extension of F-actin-mediated protrusion at the leading edge and retraction of the cell posterior margin by myosin-mediated contraction. In addition, the inflow of calcium is known to be important for cell migration. In the presence of intracellular calcium buffers, oriented directional migration occurs, but in the presence of extracellular ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EDTA), migration of chemotaxing cells toward a cyclic AMP (cAMP) source is reduced (Unterweger and Schlatterer, 1995). In addition, calciums are involved in various developmental processes, such as cell–cell adhesion and cell fate determination, and they are necessary for electrotactic cell migration (Shanley et al., 2006). These observations suggest that changes in calcium and cAMP concentrations are necessary for the development of *Dictyostelium discoideum*.

The social ameba *D. discoideum* has been used as a model system for investigating signaling pathways in eukaryotes (Chisholm and Firtel, 2004). Upon starvation,

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Dictyostelium amebae gather together and begin a multicellular developmental process, which can be divided into 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 *D. discoideum* regulates intracellular calcium concentrations during early development. The rate of Ca^{2+} influx is stimulated by the chemoattractant cAMP (Tanaka et al., 1998).

In previous studies, 14 different genes encoding small CBPs were identified in *D. discoideum*. CBP1, encoded by *cbp1*, associates with the actin cytoskeleton for cell aggregation and is expressed during multicellular stages. During development, CBP1 was first detected at ~3 h; its level increased until ~9 h, and then remained at that level until the completion of development (Dharamsi et al., 2000). The role of CBP2 (*cbp2*) is unknown, but its mRNA was shown to peak after 9 h of development and then decreased after 12 h (Andre et al., 1996). CBP3 (*cbp3*) binds to actin to associate with the actin cytoskeleton, and its mRNA is expressed at early development stages, until 12 h of development (Lee et al., 2005). CBP4a (*cbp4a*) and CBP4b (*cbp4b*) were identified as proteins interacting with nucleomorphin, which is a nuclear calmodulin-binding protein. CBP4a (*cbp4a*) was shown to play a role in regulating the number of nuclei in *D. discoideum* (Myre and O'Day, 2004). The *cbp5, cbp6, cbp7, cbp8, cbp9, cbp10, cbp11, cbp12,* and *cbp13* genes have been identified and protein products have EF-hand domains and similar small sizes, approximately 20 kDa.

To understand the function of CBP7 in diverse biological processes, I examined phenotypes of the cells expressing CBP7 or RNAi constructs in morphogenesis, growth, development, and chemotaxis. The results indicate that is involved in the regulation of morphogenesis, adhesion, growth, and development.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

II. MATERIALS AND METHODS

II-1. Cell culture and strains

The *D. discoideum* 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 *Eco*RI–*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. Expression mutants CBP7$^{N24A, D26A}$, CBP7$^{D63, 65A}$, CBP7$^{D117, 19A}$, and CBP7$^{D152, 154A}$ were generated by PCR using as the primers tj171 and tj172 for CBP7$^{N24A, D26A}$; tj169 and tj170 for CBP7$^{D63, 65A}$; tj143 and tj144 for CBP7$^{D117, 19A}$; tj145 and tj146 for CBP7$^{D152, 154A}$. To obtain the CBP7$^{D117, 119,152,154A}$ mutant, CBP7$^{D117, 19A}$ was used as the template and tj145/tj146 as the primers. The resultant point-mutagenesis fragments were ligated into the *Eco*RI–*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 *Eco*RI–*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 *Eco*RI. The short 5'-fragment of CBP7 was synthesized using primer MR5, tj67 and cloned into tj66/MR4 cloned vector.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

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

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

II-2. Cell adhesion assay

Log-phase cells growing on plates were washed with Na/K phosphate buffer and resuspended at a density of 2×10^6 cells/mL. Cells (4×10^5 in 200 μL) were plated onto 13-mm circular nitrocellulose filters (Millipore). After 30 min, unattached cells were removed by dipping filters into Na/K phosphate buffer. The filters were transferred to micro centrifuge tubes containing 800 μL of Na/K phosphate buffer and vortexed for 1 min. Cells (150 μL) detached from the filters were plated onto a 30-mm Petri plate with a hole covered by a 0.17-mm glass coverslip, and an additional coverslip was placed on top. The cells were photographed and counted (detached cell count). To determine the total cell count, 200 μL of the cells was transferred into microcentrifuge tubes containing 600 μL of Na/K buffer and counted. Cell adhesion was presented as a percentage of detached cells relative to total cells. This experiment was repeated at least three times, each time with four filters for each strain.

II-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/cm^2. 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.

II-4. Chemotaxis

Chemotaxis toward cAMP and changes in the subcellular localization of proteins in response to chemoattractant stimulation were examined as described previously (Jeon et

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

al., 2007; Sasaki et al., 2004). Aggregation-competent cells were prepared by incubating 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 (SVDCC100, UK). Images of chemotaxing cells were taken at time-lapse intervals of 6 s for 30 min using an inverted microscope. The data were analyzed using the NIS-Elements software (Nikon).

II-5. Cell sorting assay

For examination of cell movement in a multicellular organism, 2.5% of red fluorescent protein (RFP)-labeled wild-type cells and 2.5% of GFP-labeled CBP7 cells were mixed with 95% of unlabeled wild-type cells and developed on Na/K phosphate agar plates for 6 h. 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).

II-6. Electrotaxis

Dictyostelium amebae were starved in DB buffer for 1 h and seeded into an electrotaxis chamber as described previously (Zhao et al., 1996). Before electric field stimulation, two agar–salt bridges were prepared of no less than 15 cm long. The two agar–salt bridges were used to connect $AgCl_2$ electrodes in beakers with Steinberg's solution to pools of buffer at either sides of the chamber. For electric field application, the electric field strength of 20 V/cm was used. The field strength was measured at the beginning and end of the experiment (Shanley et al., 2006) .

II-7. Measurement of cytosolic calcium

Dictyostelium cells were prepared at a density of 5×10^6 cells/mL in 1 x PBS buffer,

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

and cells incubate in the dark at room temperature for 60 minutes with 2.2 mM Fluo-4 AM dissolved in dimethyl sulfoxide (final concentration 0.05%). After incubation, the cells were centrifuged (1500 g, 4 minutes, 22 °C) and washed twice with 1 x PBS buffer. The cells were placed in 96 wells plate. Set the SoftMax Pro excitation/emission wavelengths (using fluo-4, set Molecular Devices optics for excitation at 494 nm and emission at 506 nm).

II-8. Yeast two-hybrid analysis

The Gal4 yeast two-hybrid screening was used to detect protein-protein interactions. The yeast strain EGY48. The *Dictyostelium ras* cDNA was cloned into the *Eco*RI/*Xho*I site of the Gal4 activation domain (AD) vector pEG202. The *Dictyostelium* cDNA library was cloned into the *Eco*RI/*Xho*I site of the Gal4 DNA binding domain (DBD) vector pJG4-55 to obtain pJG–CBP7. Transformants were selected in a minimal medium lacking uracil, histidine, and tryptophan. The AD plasmid of pEG-Ras and DBD plasmid of pJG–CBP7 were co-transformed into yeast strain EGY48 and assayed for β-galactosidase activity. pJG–RA1 was used as a positive control for RasG interactions.

II-9. Akt/PKB kinase activity assay

Aggregation-competent cells were prepared by incubating cells at a density of 5×10^6 cells/mL in Na/K phosphate buffer for 10 h. Aggregation-competent cells treated with 1mM caffeine at room temperature for 30 min. Following treatment, cells were washed with ice-cold Na/K phosphate buffer once and resuspend with 2 ml of Na/K phosphate buffer. Then cells were stimulated with 1 mM cAMP and immediately lysed by 2 x lysis buffer [20 mM Tris pH 6.8, 1% Triton X-100, 2 mM $MgCl_2$, 5 mM EGTA] at 10, 20, and 40 s. Different time points after stimulation were added in 2 x SDS-sample buffer,

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

and equal amounts of total protein were separated on SDS–PAGE. Phosphorylation of Akt/PKB was detected using phospho-Akt Substrate antibody (Cell Signaling Technology, Danvers, MA). Coomassie Blue staining of total proteins was used as loading control. The Western blots were quantified using Image J.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III. RESULTS

III-1. Identification of the gene encoding CBP7

There are 14 genes encoding CBP proteins in the *Dictyostelium* genome sequence (Table 1). Among them, CBP1, CBP3, CBP4a, and CBP4b have been characterized, and their functions have been studied in various biological processes (Table 1). 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 19.3kDa), EF-hands 1 and 2 at the N-terminal region and EF-hands 3 and 4 at the C-terminal region (Fig. 1A). The phylogenetic trees with Calmodulin (CaM) and CBP proteins containing four EF-hands show that CBP7 is more closely related to the CBP6, CBP12 and, CBP3 (Fig. 1C).

To investigate the similarity of CBP7 to other CBP proteins, the CBP7 amino acid sequence was compared with those of CBP1, CBP2, CBP3, CBP4, CBP5, CBP6, CBP8, and CaM by multiple alignments (Fig. 2). Results showed that CBP7 had 68%, 74%, and 70% identity with CBP3, CBP6, and CBP12, respectively, and contained the amino acids necessary for calcium binding.

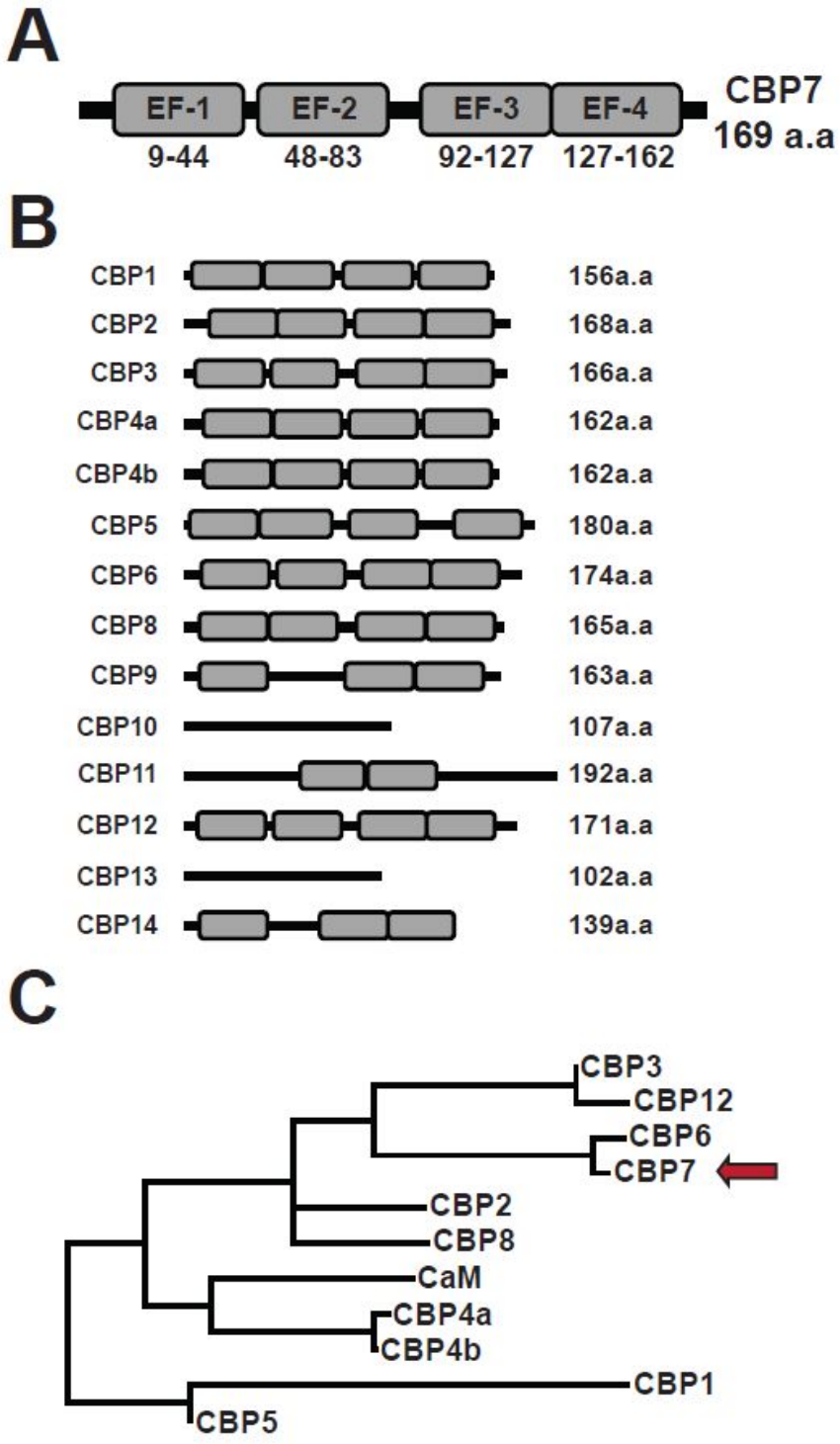
Collection @ chosun

Table2. Overview of calcium binding proteins in *Dictyostelium*

Protein	Protein size	Location	EF-hand Domains	Molecular function	Disruption	Expressed
CBP1	156 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	165 a.a	Chromosome 6	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	-	-

조선대학교 CHOSUN UNIVERSITY
Collection @ chosun

조선대학교
CHOSUN UNIVERSITY



Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Fig 1. Domain structure and phylogenetic tree of CBP proteins in *Dictyostelium*.

(A) Domain structure of CBP7 showing four EF-hands. (B) Domain structure of 13CBPs in *Dictyostelium*. (C) Phylogenetic tree analysis of CBPs. The amino acid sequence of CBP7 was compared with those of other Calcium binding proteins. *Dictyostelium discoideum* (G0276759); CBP1, (G0267456); CBP2, (G0283613); CBP3, (G0283153); CBP4a, (G0283083); CBP4b, CBP5 (G0274099); CBP5, (G0283611); CBP6, (G0283609); CBP7, (G0288623); CBP8, (G0283645); CBP12, and (G0279407); CaM. These sequences are available at www.dictybase.org.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

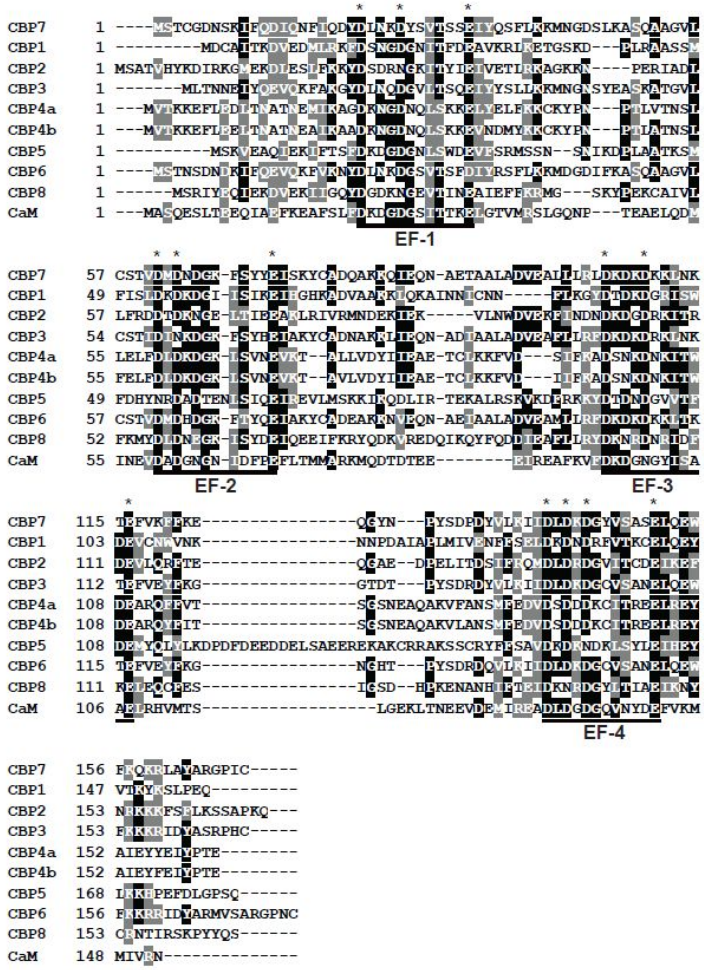


Fig 2. Multiple alignment of CBP proteins.

The amino acid sequence of CBP7 was compared with those of other calcium binding proteins, which are known as proteins including real calcium binding that CBP1, CBP2, CBP3, CBP4a, CBP4b, CBP5, CBP6, and CaM. The putative Ca^{2+}-binding regions, including four EF-hands, are shown; identical amino acid residues are highlighted in dark gray, and conserved residues are highlighted in light grey. The residues conserved in the EF-hands of eight or more proteins are marked with asterisks.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-2. CBP7 is involved in the regulation of morphogenesis

To investigate the functions of CBP7, I prepared cells expressing Myc-CBP7 (Myc-CBP7), GFP-CBP7 (GFP-CBP7), and CBP7 RNA interference constructs (CBP7-RNAi) and examined the morphology of the cells (Fig. 3A). Myc-CBP7 cells and GFP-CBP7 cells were more spread and flattened than wild-type and CBP7-RNAi cells. Measurement of cell areas using the NIS-Element software showed that Myc-CBP7 cells and GFP-CBP7cells were 1.4-fold larger than wild-type and CBP7-RNAi cells (Fig. 3B). In addition, I investigated cell adhesion of Myc-CBP7 cells and GFP-CBP7 cells by measuring the fraction of cells that detached from a plate during agitation. Compared to wild-type cells, Myc-CBP7 cells and GFP-CBP7 cells showed increased cell–substrate adhesion (Fig. 3C). On the other hand, cell adhesion of CBP7-RNAi cells was similar to that of wild-type cells. I then investigated the effect of CBP7 on the growth rate (Fig. 3D). The mean growth rate of CBP7-overexpressing cells was lower than that of wild-type cells, suggesting that CBP7 affected the growth rate. However, CBP7-RNAi cells were similar to wild-type cells in their growth rate. These results indicate that CBP7 is involved in the regulation of morphogenesis, cell adhesion, and growth.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

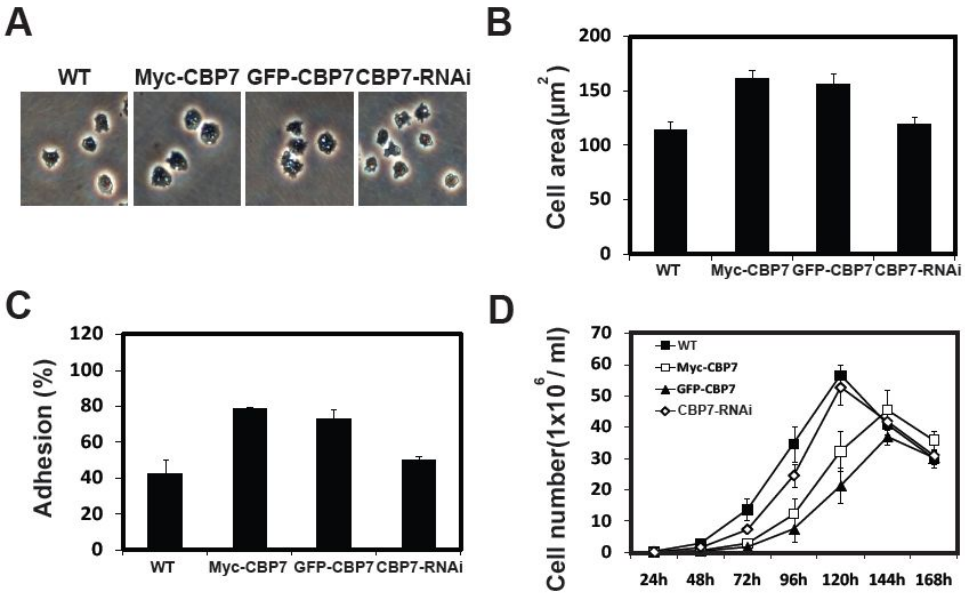


Fig 3. Phenotypes of CBP7- overexpressing cells.

(A) Morphology of CBP7 cells. (B) Measurement of cell areas of CBP7 cells using NIS-element software. (C) Cell-substratum adhesion. Adhesion was measured by the ratio of attached cells to the total number of cells. Experiments were performed at least three times. (D) Growth rates of the cells in suspension culture. Wild-type cells, Myc-CBP7 cells, GFP-CBP7 cells, and CBP7-RNAi cells were transferred from the growing plates into axenic shaking culture medium and then counted at intervals thereafter. The plotted values are the means of cell counts. From at least three independent experiments. Error bars represent SD.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-3. 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 (Chisholm and Firtel, 2004). During development, the extracellular Ca^{2+} level and the rate of Ca^{2+} influx are stimulated by chemoattractants in *D. discoideum* (Tanaka et al., 1998). To examine the possible roles of CBP7 in development, I performed a developmental assay (Fig. 4A). 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. 4A). 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. 4A). 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. 4B). These results showed that overexpression of CBP7 caused developmental defects.

To further investigate the impairment of the aggregation stage in Myc-CBP7/GFP-CBP7 cells, I examined the aggregation ability in buffer and shaking cultures (Fig. 5). In buffer culture, initial aggregation of wild-type cells and CBP7-RNAi cells started from an aggregation center within 6 h, and aggregation was completed within 10 h. However, Myc-CBP7/GFP-CBP7 cells did not initiate the developmental process (Fig. 5A). In shaking culture, wild-type cells and CBP7-RNAi cells began to aggregate within 6 h and were completely aggregated within 10 h, but Myc-CBP7 cells and GFP-CBP7 cells did not initiate aggregation (Fig. 5B). These data indicate that

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

overexpression of CBP7 results in defects in the aggregation stage.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

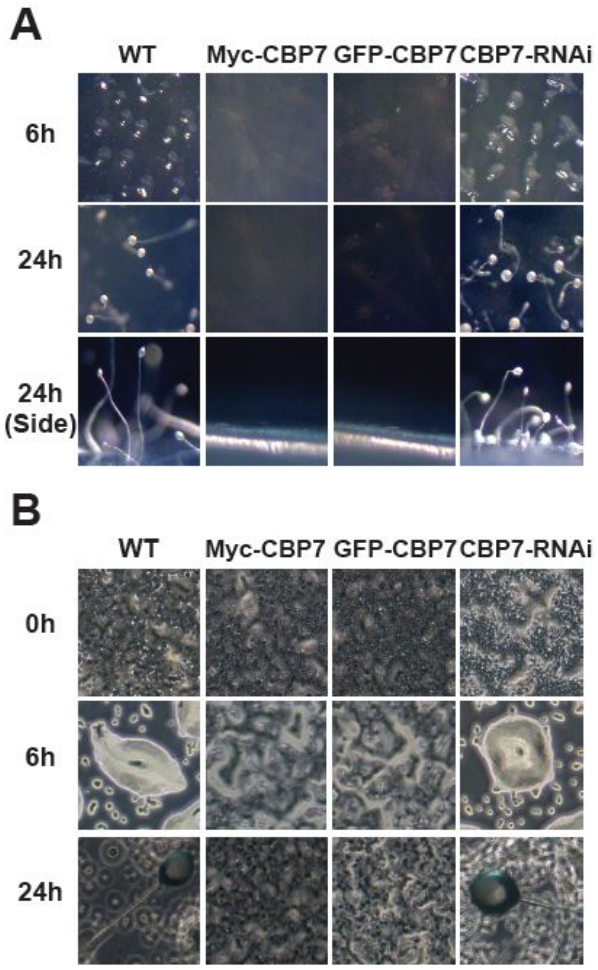
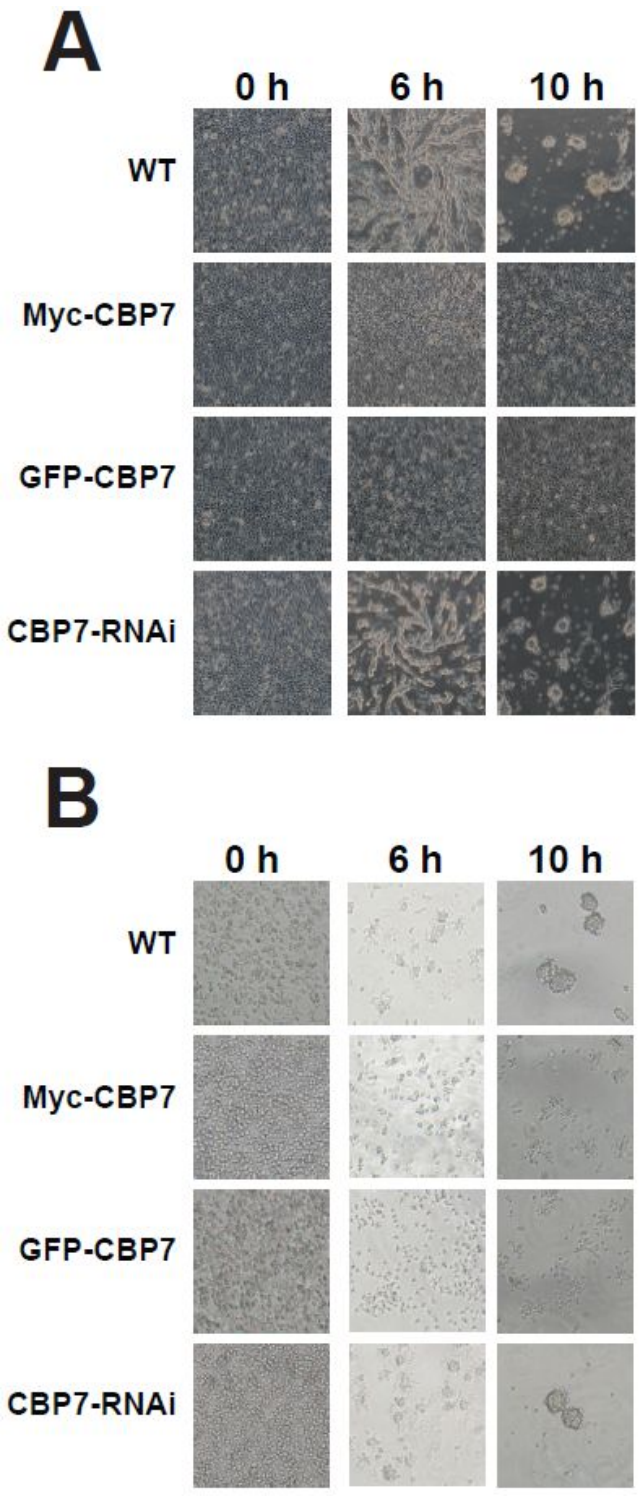


Fig 4. Development of CBP7-overexpressing cells.

(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.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY



Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Fig 5. Development of CBP7-overexpressing cells in buffers.

(A) Aggregation on a plastic surface of wild-type cells, Myc-CBP7 cells, GFP-CBP7 cells, and CBP7-RNAi cells in Na/K buffer. Vegetative cells were washed and plated on Plate (35 mm x 10 mm). Photographs were taken at the indicated times after starvation. (B) Starvation of the cells in suspension culture. Vegetative cells were washed and transferred into shacking culture medium, 12 mM Na/K phosphate buffer (pH 6.1). Photographs were taken at the indicated times after plating.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-4. Effect of overexpression of CBP7 on cell migration

1) CBP7-overexpressing cells exhibit chemotactic response defects

The previous developmental assay showed developmental defects in Myc-CBP7/GFP-CBP7 cells. In shaking culture, wild-type cells and CBP7-RNAi cells began to aggregate within 6 h and were completely aggregated within 10 h, but Myc-CBP7/GFP-CBP7 cells did not initiate aggregation (Fig. 5B). These data indicate that overexpression of CBP7 results in defects in the aggregation stage. GFP-CBP7 cells (Fig. 4). The following confirmation experiments showed that the CBP7 overexpressing cells completely lost the aggregation ability (Fig. 5). In *Dictyostelium*, initial aggregation starts by secretion of a chemoattractant, and cells move toward the chemoattractant source (Schaap et al., 1984).

Taken together, it is postulated that CBP7 might be involved in the regulation of cell migration and developmental process. Therefore, I examined the ability of CBP7-overexpressing cells to be polarized and migrated into the center of the aggregates during development. Development of chimeric cells, containing 95% unlabeled wild-type cells, 2.5% RFP-EXP expressing wild-type cells and 2.5% GFP-CBP7 expressing cells, were induced by removing the nutrients, and then the migration speed of the labeled cells were measured in the aggregation stage of development (Fig. 6A). The wild-type cells exhibited a moderate moving speed (4.53 μm/min), but it was significantly higher than GFP–CBP7 cells (2.03 μm/min) in the aggregation stage at 6 h (Fig. 6B), indicating that CBP7 plays some roles in forming aggregates by chemotaxing into the center of aggregation. The chimeric cells completed the developmental process finally forming fruiting bodies (Fig. 8). Even though CBP7 overexpressing cells showed decreased moving speed in the aggregation stage of development, fluorescence-labeled wild-type and GFP–CBP7 cells exhibited a similar distribution pattern during

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

development. Wild-type and GFP–CBP7 cells were scattered randomly throughout hemispherical aggregates (~10 h).

To determine whether CBP7-overexpressing cells are defective in cAMP-mediated chemotaxis, I compared the chemotaxis of wild-type and GFP–CBP7 cells (Fig. 7). Aggregation-competent wild-type and GFP–CBP7 cells were prepared by starving the cells in Na/K phosphate buffer for 10 h. In response to cAMP, wild-type and GFP–CBP7 cells moved at 8.93 μm/min and 8.28 μm/min, respectively, toward the cAMP source. However, the directionality of GFP–CBP7 cells, which is a measure of how straight the cells move, was lower than that of wild-type cells (Fig. 7C). These data suggest that CBP7 is involved in directional sensing during chemotaxis.

2) CBP7 is not involved in electrotaxis

It is known that calcium influx is required for chemotaxis as well as electrotaxis in *Dictyostelium* (Firtel and Chung, 2000; Zhao et al., 2002). To investigate the possible roles of CBP7 in electrotaxis, I compared the directedness and velocity of wild-type cells and GFP–CBP7 cells (Fig. 9). The directionality and moving speed in electrotaxis were all similar in both strains, wild-type cells and GFP–CBP7 cells. These results indicate that overexpression of CBP7 does not affect electrotaxis ability of the cells.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

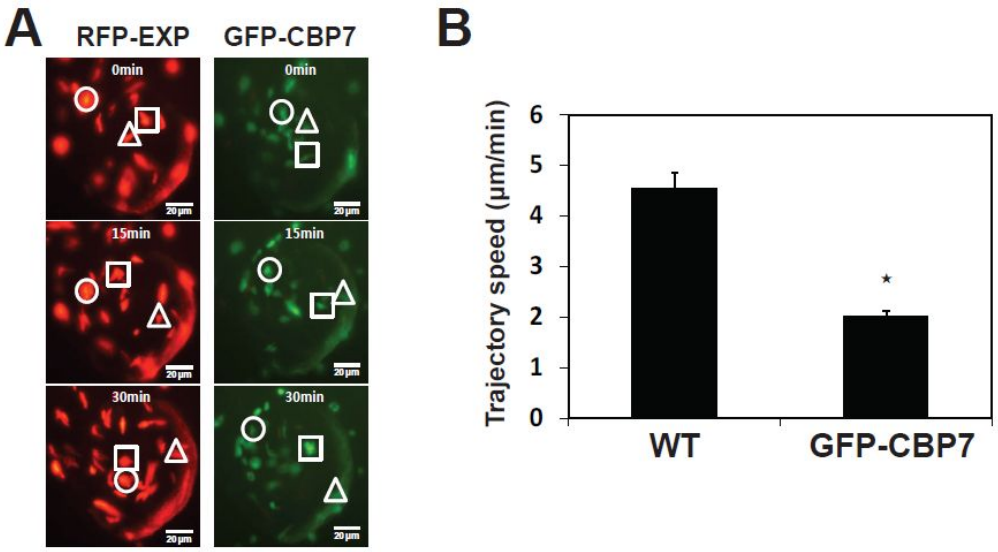


Fig 6. Analysis of cell motility during development.

(A) Development of the chimeric cells. 2.5% RFP-labeled wild-type cells and 2.5% GFP-labeled CBP7 cells were mixed with unlabeled 95% wild-type cells and developed on non-nutrient agar plates around for 6 h. Time-lapse fluorescence movies were collected to assess cell motion for 30min at 1min intervals. (B) Quantification of cell motility of the chimeric cells. The recorded images were analyzed by NIS-element and Image J software. Experiments were performed at least three times. Error bars represent SE. Statistically different from control at *$p < 0.05$ by the student's t-test.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

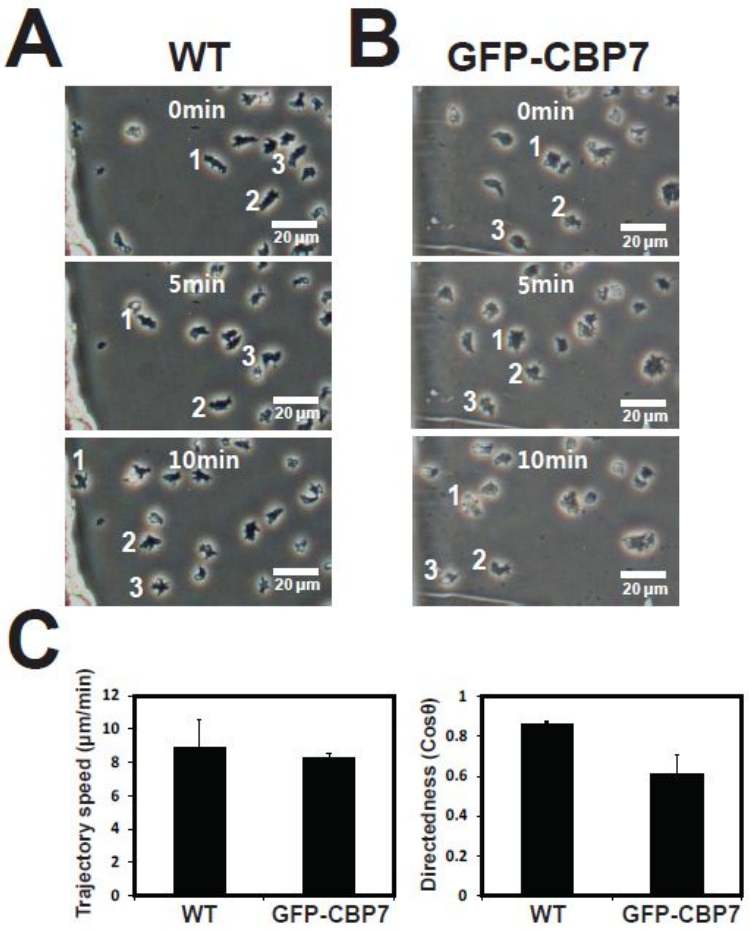
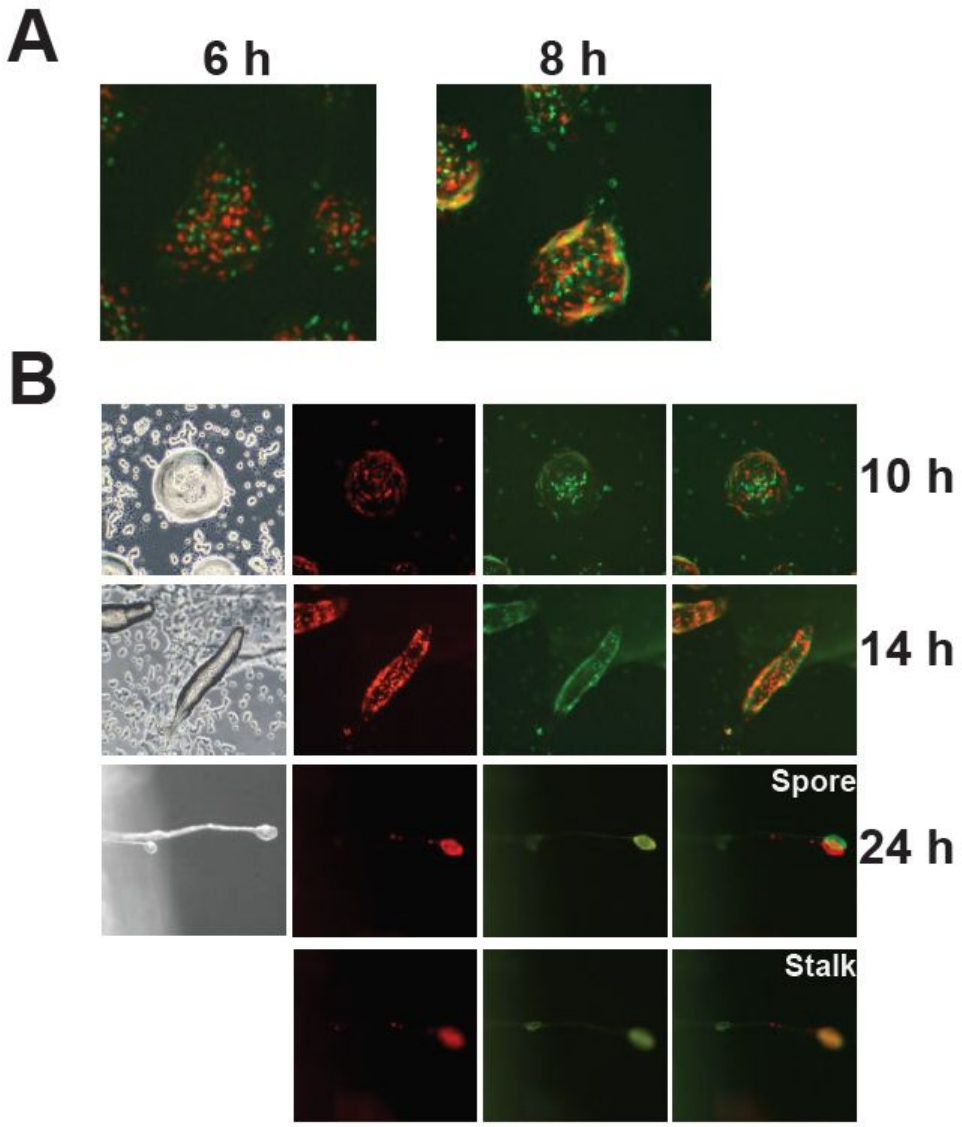


Fig 7. Analysis of cell motility in chemotaxis.

(A) Wild-type cells and GFP-CBP7 overexpressing cells (B) were placed in a Dunn chemotaxis chamber, and the movements of the cells towards cAMP gradient. (C) Quantification of cell motility of chemotaxing cells. The recorded images were analyzed by NIS-element software. Trajectory speed is the total distance travelled by a cell divided by time. A cell moving directly to the cAMP would have a directedness of 1, whereas a cell moving directly to the opposite direction of cAMP would have a direction of migration of –1. Experiments were performed at least three times. Error bars represent SD.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY



Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Fig 8. Distribution of CBP7-overexpressing cells during development

(A) Distribution of GFP-CBP7 cells and RFP-labeled wild-type cells at the aggregation stage of development. (B) Distribution of the cells at the mound, slug, and fruiting body forming stages of development. 2.5% RFP-labeled wild-type cells, 2.5% GFP-CBP7 overexpressing cells and 95% unlabeled wild-type cells were mixed and developed. The images were taken at the indicated times.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

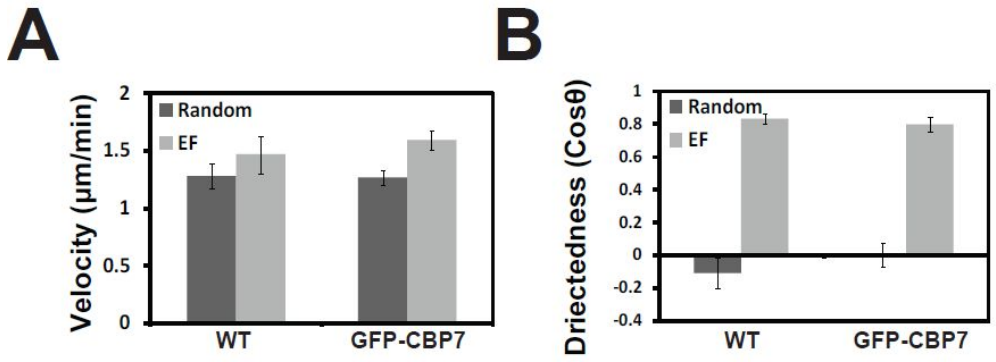


Fig 9. Analysis of cell motility in electrotaxis

(A) Velocity speeds increased significantly in both types of cells following field stimulation. (B) Wild-type and GFP-CBP7 cells migrate towards the cathode with similar direction of cell migration. The graphs show migration of cells in an electric field of 20 V/cm and take, average of 63-68 cells. The instantaneous velocity is being calculated for a particular cell and it is the time interval between frames. Directedness was assessed as cosine θ, a cell moving directly to the cathode would have a directedness of 1, whereas a cell moving directly to the anode would have a direction of migration of –1. Experiments were performed at least three times. Error bars represent SD.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-5. Overexpression of CBP7 decreases the cytosolic free calcium concentration

Influx of Ca^{2+} and elevation of its level by cAMP are important for cell aggregation of *Dictyostelium*. Stimulation of cells with cAMP-induced rapid influx of Ca^{2+} is a part of an important signal transduction mechanism in aggregation and development (Newell et al., 1995). In *Dictyostelium*, changes in calcium concentrations are important for developmental stages. The calcium concentration in amoebae rapidly increases when cell aggregation occurs (Tanaka et al., 1998). To investigate the inhibition of development in CBP7-overexpressing cells, I conducted an experiment to determine the cytosolic calcium concentration in CBP7-overexpressing cells using Fluo-4 AM. Wild-type cells, CBP7-RNAi cells, and Myc-CBP7 cells were harvested, and the intracellular free calcium concentrations were measured in a 96-well Molecular Devices using Fluo-4AM (Fig. 10). The intracellular free calcium levels for Myc-CBP7 cells were much lower than those for wild-type cells. These results suggest that mis-regulation of intracellular free calcium level might results in developmental defects in CBP7 overexpressing cells.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

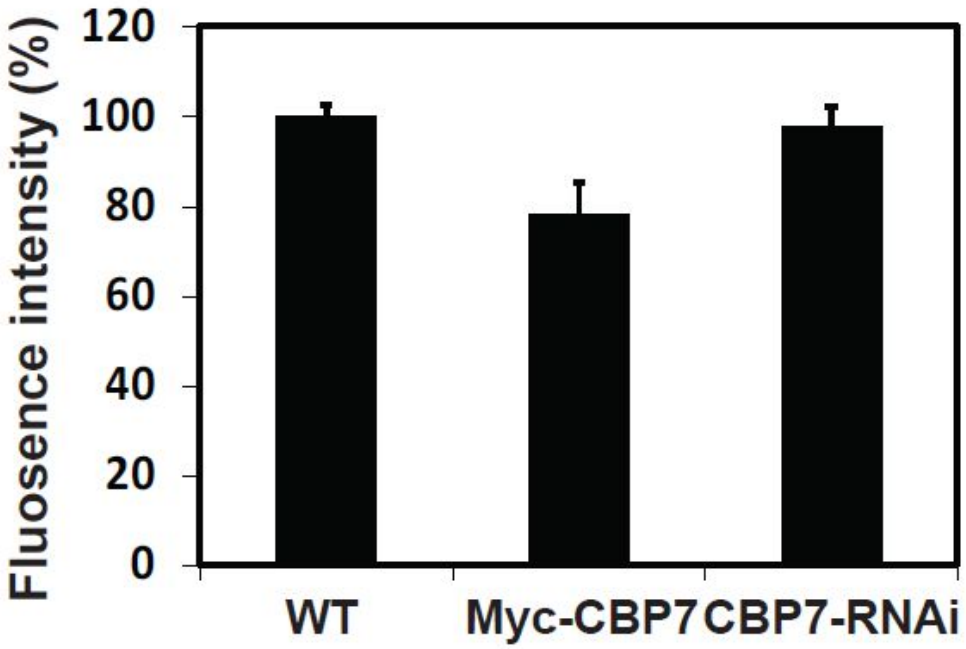


Fig 10. Measurement of the amount of intracellular free calcium.

The intracellular free calcium concentrations were measured by Molecular Devices 96 wells using Fluo4- AM (λ ex 494 nm; λ em 516 nm). Experiments were performed at least three times. Error bars represent SD.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-6. Development at various calcium concentrations

CBP7-overexpressing cells did not develop on Na/K phosphate plates (Figs. 4 and 5), and had lower intracellular free calcium concentrations (Fig. 10). 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. 11A). These results indicate that high calcium concentrations inhibit the development (Maeda, 1970). 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. 11B). 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. 11A, 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. 12A). CBP7-overexpressing cells did not develop on Na/K plates containing 0, 2, 4, and 6 mM EDTA (Fig. 12). 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.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

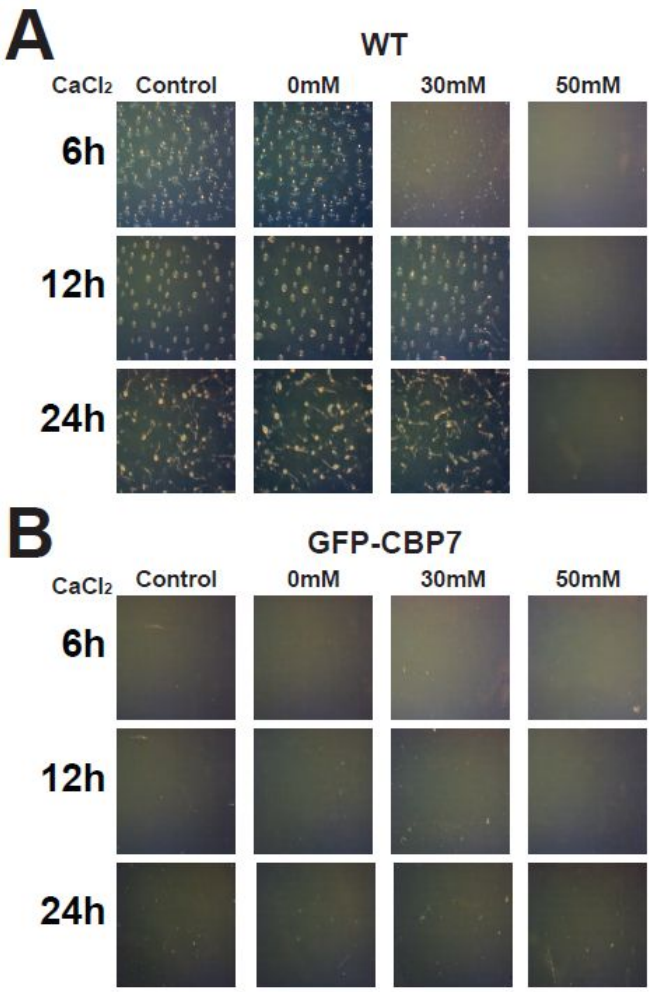


Fig 11. Developmental phenotypes of the cells at various calcium concentrations.

(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).

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

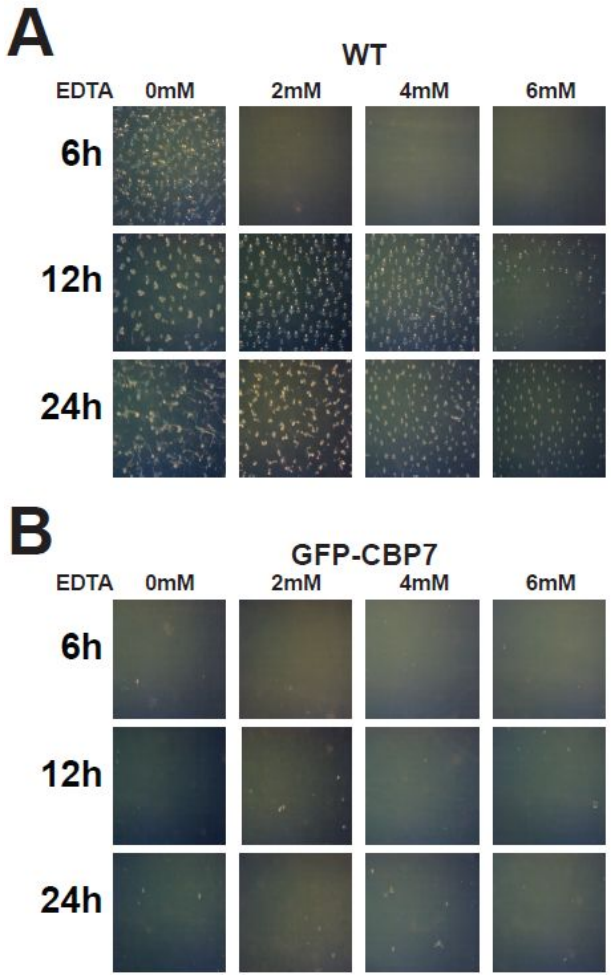


Fig 12. Developmental phenotypes of the cells in the presence of EDTA

(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).

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-7. EF-hands in CBP7 are important in the process of development

Previous sequence analysis showed homology of CBP7 to other CBP proteins (Sakamoto et al., 2003). 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 (Gifford et al., 2007; Trave et al., 1995). 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 (Mishig-Ochiriin et al., 2005). CBP7 has four putative EF-hand motifs (residues D22–E33, D70–E81, D105–E116, and D140–E151) (Fig. 1A). 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.13A). 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. 14). 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. 14B). These results indicate that the EF-hand 3 and 4 domains play an important role in development.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

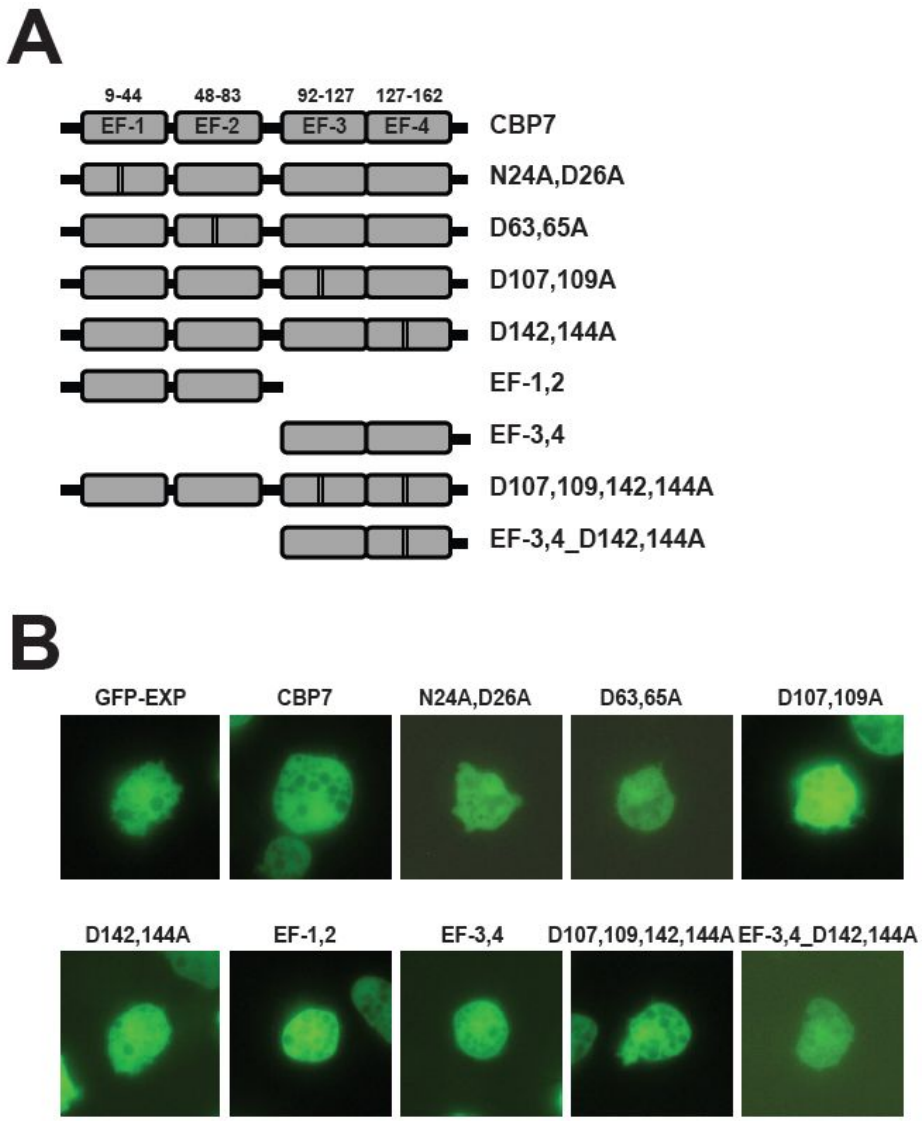
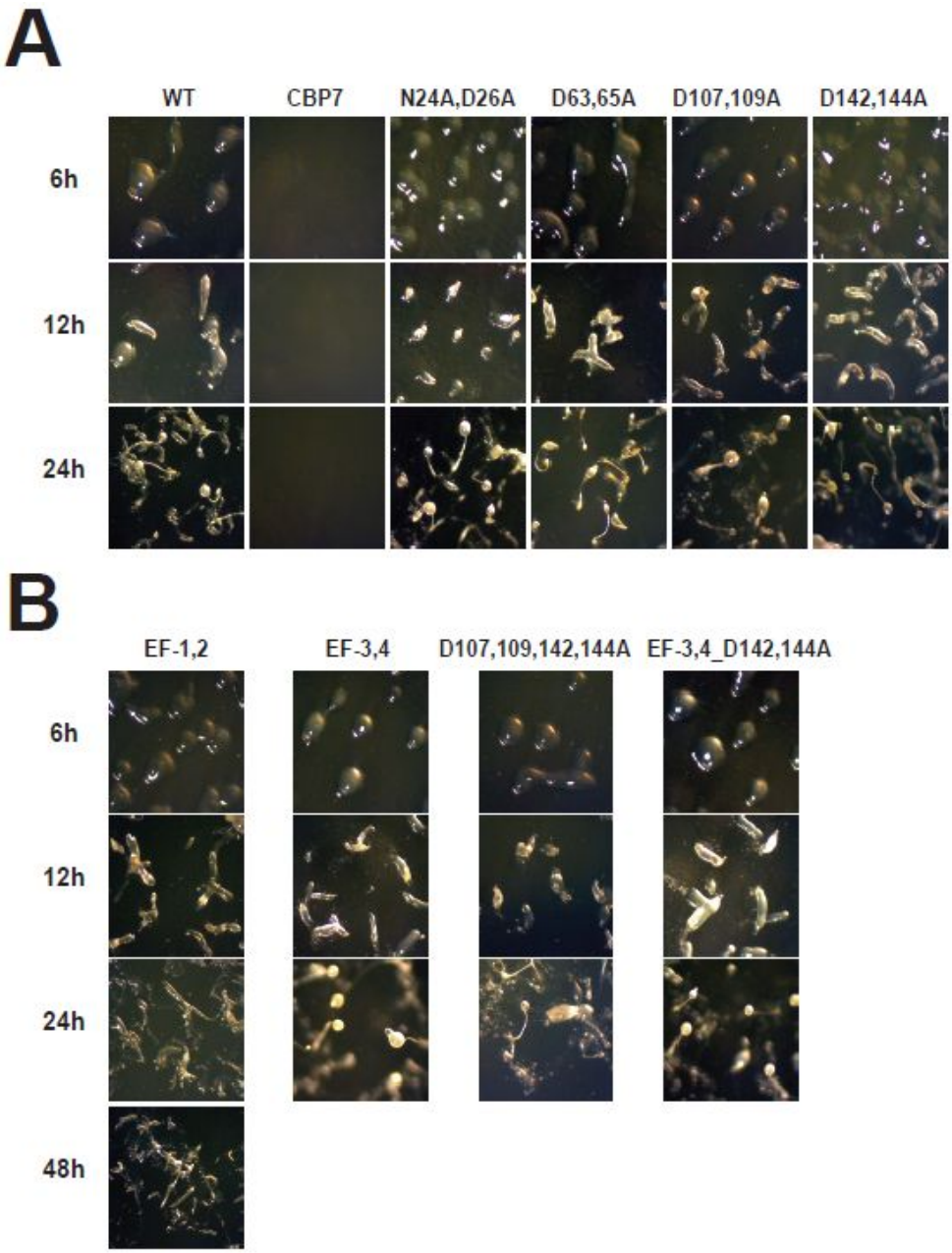


Fig 13. Domain structure and localization of point-mutated CBP7 proteins.

(A) Domain structure of the EF-hand point-mutated of CBP7 and truncated CBP7 proteins. (B) Localization of GFP-CBP7, $CBP7^{N24,D26A}$, $CBP7^{D63,65A}$ $CBP7^{D107,109A}$, $CBP7^{D142,144A}$, $CBP7^{D107,109,142,144A}$, ΔEF3,4, ΔEF1,2, $\Delta EF1,2^{D142,144A}$.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY



Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Fig 14. Development of the cells expressing point-mutated CBP7 proteins.

(A) Development of the cells expressing point-mutated CBP7 proteins. Wild-type, GFP-CBP7, CBP7$^{N24,\ D26A}$, CBP7$^{D63,\ 65A}$, CBP7$^{D107,\ 109A}$, and CBP7$^{D142,\ 144A}$. (B) Development of the cells expressing truncated CBP7 proteins. CBP7$^{D107,\ 109,142,144A}$, ΔEF3,4, ΔEF1,2, and ΔEF1,2^{D142,144A}. 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.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

III-8. CBP7 signaling pathway

In the previous experiments, CBP7 was identified as a Rap1 binding protein in yeast two-hybrid experiments. Recently, K-Ras4B, one of the mammalian Ras proteins, and the KRAS isoform were found to interact with calmodulin (Abraham et al., 2009; Nussinov et al., 2015). The Ras GTPase subfamily comprises 15 proteins in *Dictyostelium*, including 11 Ras proteins, 3 Rap proteins, and a Rheb-related protein (Kortholt and van Haastert, 2008). To determine the binding affinity to Ras proteins, I performed yeast two-hybrid experiments using CBP7 as a bait (Fig. 15). Interestingly, the results showed that CBP7 had a strong binding affinity to RasG and a weak affinity to Rap1, if any. All other Ras proteins showed no interaction with CBP7. These results suggest that CBP7 might be a downstream effector of RasG, and provide an important clue for understanding the relationship between the Ras signaling and the calcium signaling pathways.

Ras proteins have been proposed to be an activator of phosphoinositide 3-kinases (PI3Ks) (Suire et al., 2002). RasG is closely related to mammalian H-Ras and K-Ras and has a conserved effecter domain (Lim et al., 2002). *rasG*-null cells only show a partial reduction in protein kinase B (PKB, also known as Akt) activation. To determine if CBP7 is involved in the regulation of Akt/PKB activity, I examined the activation levels of the PI3K effector Akt/PKB in wild-type, GFP-CBP7, and CBP7-RNAi cells in response to cAMP chemoattractant stimulation. The chemoattractant-induced Akt/PKB activation was decreased inCBP7-RNAi cells (Fig. 16). However, chemoattractant-induced Akt/PKB activation in GFP-CBP7 cells was similar to that in wild-type cells. These results indicate that CBP7 might be involved in the regulation of Akt/PKB activation in response to chemoattractant stimulation.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

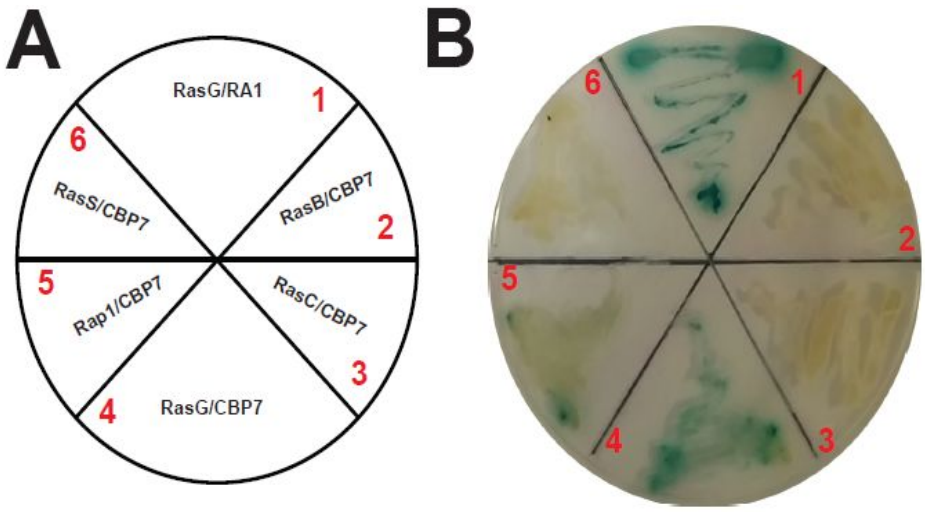


Fig 15. CBP7 binds to RasG in yeast two-hybrid analysis.

(A) Template for (B). Small scale yeast two-hybrid analysis of the interaction of RasG and Rap1 with CBP7. Yeast harboring both bait (pEG) and prey (pJG) plasmids grow in triple drop-out media (-Ura-His-Trp) and are positive for β-galactosidase. Interactions were evaluated by yeast growth on the plates lacking uracil, histidine and tryptophan. The numbers correspond to those in panel, 1: RasG / RA1, 2: RasB / CBP7, 3: RasC / CBP7, 4: RasG / CBP7, 5: Rap1 / CBP7, 6: RasS / CBP7.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

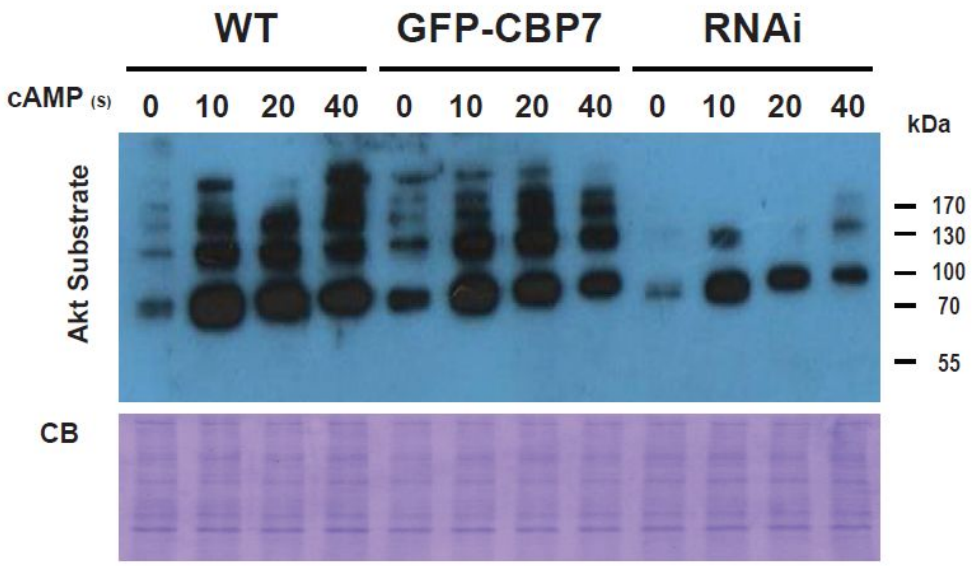


Fig 16. CBP7 is involved in the regulation of Akt/PKB activity

Wild-type cells (WT), GFP-CBP7 cells, and CBP7-RNAi cells were stimulated with 10 μM cAMP for the indicated time. Akt-substrate phosphorylations were revealed by immunoblotting with anti-Akt substrate antibodies. Equal loading was controlled with Coomassie Blue staining.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

IV. 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. 1A, B). The phylogenetic tree created be using the CLUSTAL W and Mega 5.0 programs suggested that the *Dictyostelium* CBP7 is close to CBP3, CBP6, and CBP12 (68.07%, 73.37%, and 69.82% identity, respectively).

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 prespore region. CBP8 is expressed at the slug stage. CBP3, CBP4b, CBP5, CBP6, CBP7, and CBP8 have been known as real calcium binding proteins (Sakamoto et al., 2003). 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 (Dharamsi et al., 2000). CBP3 has been known to interact with actin and is involved in slug migration (Lee et al., 2005). Moreover, CBP3 undergoes conformational changes and exposes the hydrophobic region, which results in interactions with binding partners (Mishig-Ochiriin et al., 2005). CBP4a has been known to regulate the number of nuclei in *Dictyostelium* (Myre and O'Day, 2004). In *Dictyostelium*, overexpression of chromatin assembly factor 1 (CAF1) has a stimulatory effect on differentiation, but a *caf1*-null mutant exhibited normal development (Itoh et al., 1998). It is interesting that CBP7-overexpressing cells showed somewhat opposite effects to those of CAF1-

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

overexpressing cells. Cells with CBP7-RNAi exhibited normal development. However, Myc-CBP7 and GFP-CBP7 cells exhibited strong adhesion to a substrate and failed to undergo the developmental process (Fig. 2 and 3). These data suggest that CBP7 may involve a different mechanism compared to those of other CBP proteins in the developmental process.

CBP7 is involved in chemotactic migration

Chemoattractants induce elevated transport of Ca^{2+} ions across the plasma membrane in *Dictyostelium* (Nebl and Fisher, 1997). On the other hand, a study of the inositol 1,3,5-trisphosphate receptor-like gene, *iplA*, has shown that Ca^{2+} signaling is not required for chemotaxis of *Dictyostelium* cells (Schaloske et al., 2005). Electrotaxis is not required for the *iplA* gene either (Shanley et al., 2006). However, both pathways require calcium influx.

When intracellular free calcium levels were measured, CBP7-overexpressing cells had lower calcium levels than wild-type cells (Fig. 10). Also, I observed the early aggregation stages on Na/K phosphate agar plates at a density of 3.5 × 107 cells/cm^2 and in Na/K phosphate buffer (pH 6.1), there was no cAMP pulsing (Fig. 4). It is reasonable to assume that overexpression of CBP7 results in a defect in cAMP generation or responses. In cell sorting experiments using wild-type (95%), RFP-EXP (2.5%), and GFP–CBP7 (2.5%) cells, the GFP–CBP7 cells showed a much lower speed (2.03 μm/min) than that of the wild-type cells (4.53 μm/min) (Fig. 6B). In the chemotaxis assay, wild-type and CBP7-overexpressing cells showed similar speeds. However, CBP7-overexpressing cells showed lower directedness than that of wild-type cells (Fig. 7). In an electric field, wild-type and GFP-CBP7 cells increased the velocity speed and directedness compared to those observed in the absence of an electric field (Fig. 8). Thus, it is reasonable to assume that overexpression of CBP7 may be caused defects in chemotactic migration due to calcium regulatory failure.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

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 (Gifford et al., 2007). 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. The sequence analysis showed homology between CBP7 and CBP3 (Fig. 1 and 2).

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.

CBP7 is involved in RasG signaling pathway

According to previous studies, CaM interacts with a hypervariable region of KRAS4B but not with other Ras isoforms. KRAS4B is an oncogenic splicing isoform of KRAS, which plays an important role in cell proliferation and motility in mammalian cells (Abraham et al., 2009; Nussinov et al., 2015). There are multiple Ras proteins, of which six (RasG, RasC, RasD, RasB, RasS, and Rap1) have been characterized in *Dictyostelium* (Weeks and Spiegelman, 2003). RasG is involved in cell adhesion and development. Expression of a constitutively activated version of RasG, RasG (G12T),

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

blocks the development (Khosla et al., 1996), and initiation of development is delayed in *rasG*-null cells (Tuxworth et al., 1997). CBP7, one of CBP proteins, plays an important role in the morphogenesis and development. In this study, using a small-scale yeast two-hybrid system, I provided evidence that CBP7 binds to RasG and also showed that CBP7 plays an important role in Akt/PKB activation. These results seem to be related to developmental defects induced by overexpression of CBP7.

Conclusions

CBP proteins are involved in various cellular processes, cell migration, and development in *Dictyostelium*. These CBP proteins have highly conserved EF-hand motifs, with Ca^{2+} ion binding to the interhelical loop region. Overexpression of CBP7 resulted in severe defects in diverse cellular processes including morphogenesis, adhesion, growth, chemotaxis, and development. In particular, CBP7 overexpressing cells showed complete loss of developmental process. In addition, CBP7-overexpressing cells exhibited low level of cytosolic free calcium concentration, which is important for calcium-induced developmental regulatory mechanisms. 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. Yeast two-hybrid experiments using CBP7 as a bait showed that CBP7 binds to RasG. Characterization of CBP7 in this study will contribute to understand the functions of calcium signaling and Ras signaling pathway.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

V. REFERENCES

Andre, B., Noegel, A.A., and Schleicher, M. (1996). *Dictyostelium discoideum* contains a family of calmodulin-related EF-hand proteins that are developmentally regulated. FEBS letters *382*, 198-202.

Chin, D., and Means, A.R. (2000). Calmodulin: a prototypical calcium sensor. Trends in cell biology *10*, 322-328.

Chisholm, R.L., and Firtel, R.A. (2004). Insights into morphogenesis from a simple developmental system. Nature reviews. Molecular cell biology *5*, 531-541.

Dharamsi, A., Tessarolo, D., Coukell, B., and Pun, J. (2000). CBP1 associates with the Dictyostelium cytoskeleton and is important for normal cell aggregation under certain developmental conditions. Experimental cell research *258*, 298-309.

Firtel, R.A., and Chung, C.Y. (2000). The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients. BioEssays : news and reviews in molecular, cellular and developmental biology *22*, 603-615.

Gifford, J.L., Walsh, M.P., and Vogel, H.J. (2007). Structures and metal-ion-binding properties of the Ca2+-binding helix-loop-helix EF-hand motifs. The Biochemical journal *405*, 199-221.

Itoh, M., Noguchi, M., and Maeda, Y. (1998). Overexpression of CAF1 encoding a novel Ca2+-binding protein stimulates the transition of Dictyostelium cells from growth to differentiation. Development, growth & differentiation *40*, 677-683.

Jeon, T.J., Lee, D.J., Merlot, S., Weeks, G., and Firtel, R.A. (2007). Rap1 controls cell adhesion and cell motility through the regulation of myosin II. The Journal of cell biology *176*, 1021-1033.

Khosla, M., Spiegelman, G.B., and Weeks, G. (1996). Overexpression of an activated rasG gene during growth blocks the initiation of Dictyostelium development. Molecular and cellular biology *16*, 4156-4162.

Lee, C.H., Jeong, S.Y., Kim, B.J., Choi, C.H., Kim, J.S., Koo, B.M., Seok, Y.J., Yim, H.S., and Kang, S.O. (2005). Dictyostelium CBP3 associates with actin cytoskeleton and is related to slug migration. Biochimica et biophysica acta *1743*, 281-290.

Lee, M.R., and Jeon, T.J. (2012). Cell migration: regulation of cytoskeleton by Rap1 in Dictyostelium discoideum. J Microbiol *50*, 555-561.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Lee, W.H., Kim, S.H., Jeong, E.M., Choi, Y.H., Kim, D.I., Lee, B.B., Cho, Y.S., Kwon, B.S., and Park, J.E. (2002). A novel chemokine, Leukotactin-1, induces chemotaxis, pro-atherogenic cytokines, and tissue factor expression in atherosclerosis. Atherosclerosis *161*, 255-260.

Lim, C.J., Spiegelman, G.B., and Weeks, G. (2002). Cytoskeletal regulation by Dictyostelium Ras subfamily proteins. Journal of muscle research and cell motility *23*, 729-736.

Marks, P.W., and Maxfield, F.R. (1990). Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. The Journal of cell biology *110*, 43-52.

Mishig-Ochiriin, T., Lee, C.H., Jeong, S.Y., Kim, B.J., Choi, C.H., Yim, H.S., and Kang, S.O. (2005). Calcium-induced conformational changes of the recombinant CBP3 protein from Dictyostelium discoideum. Biochimica et biophysica acta *1748*, 157-164.

Myre, M.A., and O'Day, D.H. (2004). Dictyostelium calcium-binding protein 4a interacts with nucleomorphin, a BRCT-domain protein that regulates nuclear number. Biochemical and biophysical research communications *322*, 665-671.

Nebl, T., and Fisher, P.R. (1997). Intracellular Ca2+ signals in Dictyostelium chemotaxis are mediated exclusively by Ca2+ influx. Journal of cell science *110 (Pt 22)*, 2845-2853.

Sakamoto, H., Nishio, K., Tomisako, M., Kuwayama, H., Tanaka, Y., Suetake, I., Tajima, S., Ogihara, S., Coukell, B., and Maeda, M. (2003). Identification and characterization of novel calcium-binding proteins of Dictyostelium and their spatial expression patterns during development. Development, growth & differentiation *45*, 507-514.

Sasaki, A.T., Chun, C., Takeda, K., and Firtel, R.A. (2004). Localized Ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. The Journal of cell biology *167*, 505-518.

Schaap, P., Konijn, T.M., and van Haastert, P.J. (1984). cAMP pulses coordinate morphogenetic movement during fruiting body formation of Dictyostelium minutum. Proceedings of the National Academy of Sciences of the United States of America *81*, 2122-2126.

Schaloske, R.H., Lusche, D.F., Bezares-Roder, K., Happle, K., Malchow, D., and Schlatterer, C. (2005). Ca2+ regulation in the absence of the iplA gene product in

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

Dictyostelium discoideum. BMC cell biology *6*, 13.

Shanley, L.J., Walczysko, P., Bain, M., MacEwan, D.J., and Zhao, M. (2006). Influx of extracellular Ca2+ is necessary for electrotaxis in Dictyostelium. Journal of cell science *119*, 4741-4748.

Suire, S., Hawkins, P., and Stephens, L. (2002). Activation of phosphoinositide 3-kinase gamma by Ras. Current biology : CB *12*, 1068-1075.

Tanaka, Y., Nakayama, K., and Shigenobu, K. (1998). Changes in cytosolic Ca2+ measured by use of fura-2 and contraction produced by quick stretch and various stimulants in canine cerebral artery. Research communications in molecular pathology and pharmacology *102*, 79-92.

Tuxworth, R.I., Cheetham, J.L., Machesky, L.M., Spiegelmann, G.B., Weeks, G., and Insall, R.H. (1997). Dictyostelium RasG is required for normal motility and cytokinesis, but not growth. The Journal of cell biology *138*, 605-614.

Unterweger, N., and Schlatterer, C. (1995). Introduction of calcium buffers into the cytosol of Dictyostelium discoideum amoebae alters cell morphology and inhibits chemotaxis. Cell calcium *17*, 97-110.

Van Haastert, P.J. (1995). Transduction of the chemotactic cAMP signal across the plasma membrane of Dictyostelium cells. Experientia *51*, 1144-1154.

Weeks, G., and Spiegelman, G.B. (2003). Roles played by Ras subfamily proteins in the cell and developmental biology of microorganisms. Cellular signalling *15*, 901-909.

Zhao, M., Jin, T., McCaig, C.D., Forrester, J.V., and Devreotes, P.N. (2002). Genetic analysis of the role of G protein-coupled receptor signaling in electrotaxis. The Journal of cell biology *157*, 921-927.

Collection @ chosun

조선대학교
CHOSUN UNIVERSITY

VI. Acknowledgements

대학원에 입학 한지 엊그제 같은데 학위 논문 심사를 다 마치고 벌써 졸업을 앞두고 있습니다. 먼저, 실험실 생활이 처음에 낯설고 어려웠지만 같이해준 여러 실험실 식구들과 2 년 동안 부족한 저를 믿고 지금까지 옆에서 도와주신 전택중 교수님께 감사 드리며, 논문 심사 해주신 조광원 교수님, 송상기 교수님 감사 드립니다. 많은 지도와 가르침 주신 박현용 교수님, 조태오 교수님, 이준식 교수님 감사 드립니다.

처음 접해보는 실험실 생활이 어색하고 실험결과가 뜻대로 안 나와서 많이 속상하고 아쉬운 점이 많았지만 옆에서 여러 도움을 주신 분들이 있어 학위 생활을 무사히 마칠 수 있었습니다. 제 나름대로는 열심히 했지만 실험을 하는 중에 많은 여러 어려움이 있을 때마다 조언을 해준 미래, 혜선이 고맙다. 실험실에서 힘든 일을 겪을 때 항상 고민 들어준 병규, 평화야 고마워. 고등학교 졸업 후에 서로 바빠서 1 년에 한두 번 만나지만, 만날 때마다 항상 힘이 되어준 용석, 홍문 고마워. 같이 대학원 입학해서 석사과정을 마무리하는 호태, 민지, 푸름, 혜선이 고생했어. 박사과정 졸업하는 신구형 정말 수고하셨습니다. 마지막으로, 2 년 동안 걱정 없이 석사까지 할 수 있게 뒷바라지 해주신 형 과 부모님 감사합니다. 모두 언급은 못했지만 모두들 감사했습니다. 2 년 동안 배운 경험들 잊지 않고 앞으로 더 노력해서 어디 실험실로 가든지 잘해내겠습니다.

Collection @ chosun