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Reversible function of RapA with the C-terminal region of RapC and development of *rapC* null cells in *Dictyostelium*

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딕티오스텔리움에서 RapC C-말단 부위를 이용한 RapA 기능 변화 및 *rapC* 결핍 세포주의 발생 연구

2021 년 2 월 25 일

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이 논문을 이학석사학위 신청 논문으로 제출함

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ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
DAPI	4', 6'-diamidine-2'-phenylidole dihydrochloride
DIF	Differentiation inducing factor
GAP	GTPase activating proteins
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
mTOR	Mechanistic target of rapamycin
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PI3K	Phosphatidylinositol 3-kinase
RFP	Red fluorescent protein
SD	Standard denation

ABSTRACT

Reversible function of RapA with the C-terminal region of RapC and development of *rapC* null cells in *Dictyostelium*

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Ras proteins are small GTPases involved in diverse signaling pathways including cell migration and differentiation. RapA is a key regulator of migration and cell adhesion in *Dictyostelium*. Recently it has been reported that loss of RapC, which is a Ras subfamily protein with the highest homology to RapA, resulted in severe defects in multicellular development and migration. Interestingly, these phenotypes of *rapC* null cells are similar to those of the cells expressing the constitutively activated form of RapA, suggesting that RapA and RapC might play opposite functions in cell spreading and cell migration. RapC has a unique long stretched C-terminus, which is not present in RapA and other Ras proteins, and it was postulated that the C-terminus of RapC might play some roles in the antagonistic effects of RapC to RapA. To determine the functions of the C-terminus of RapC, I examined if the phenotypes of *rapC* null cells were rescued by expressing full-length RapC or the C-terminus deleted RapC. Only the intact RapC were able to rescue the defects of *rapC* null cells. In addition, I examined whether the C-terminus of RapC

plays a role in reversing the functions of RapA using a recombinant RapA fused with the C-terminus of RapC. Recombinant RapA fused with the C-terminus of RapC completely recovered the phenotypes of *rapC* null cells, indicating that the functions of RapA were modified to become similar to those of RapC by the C-terminus of RapC with respect to cell morphology, cell adhesion and migration, cytokinesis, and development.

Dictyostelium cells feed on bacteria. When all the food is exhausted, the cells undergo developmental processes; *Dictyostelium* cells secrete chemoattractants to communicate with each other enabling them to form a multicellular aggregate. The aggregates transform to a motile slug which later culminates to a fruiting body with a dead stalk holding a mass of dormant spores. The previous study showed that RapC was required for proper development, and *rapC* null cells had developmental phenotypes forming multiple tips from a single mound and multi-branched developmental structures. To investigate the reasons for the developmental defects of cells lacking RapC, I determined if the cAMP signaling pathway and membrane potential are involved in development. Caffeine is known as an antagonist of adenosine and suppresses vibrating cAMP waves. When *Dictyostelium* cells were developed in the presence of caffeine, interestingly the developmental phenotypes, forming multiple tips, similar to those of *rapC* null cells were observed. These results suggest that the cAMP signaling pathway might be defective in *rapC* null cells causing the developmental phenotypes. To determine if the membrane potential is involved in development, I examined development at the various concentrations of potassium in the developmental media. There was no significant difference in development at high concentrations of potassium compared to wild-type cells. However, multi-tips were formed when the cells were applied with electric fields in the presence of high concentrations of potassium. Further studies should be followed for understanding the mechanism for multi-tip formation during development and the Ras signaling pathway.

국문초록

딕티오스텔리움에서 RapC C-말단 부위를 이용한 RapA 기능 변화 및 *rapC* 결핍 세포주의 발생 연구

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Ras 단백질은 세포 이동 및 분화를 포함한 다양한 신호 전달 경로에 관여하는 작은 GTPase이다. RapA는 딕티오스텔리움에서 세포 부착 및 이동에 중요한 역할을 한다고 알려져 있다. 최근 논문에서는 RapA와 가장 높은 상동성을 갖는 Ras 서브 패밀리 단백질 인 RapC의 손실이 발달 과정 및 이동에 심각한 결함을 초래한다는 것이 알려졌다. 흥미롭게도 이러한 *rapC* null 세포의 표현형은 RapA를 발현하는 세포의 표현형과 유사하며, 이는 RapA와 RapC가 세포 확산 및 세포 이동에서 반대 기능을 할 수 있음을 시사합니다. 또한 RapC는 다른 Rap 단백질에 존재하지 않는 고유 한 긴 C- 말단을 가지고 있다. RapC의 C-말단의 기능을 결정하기 위해, 우리는 RapC를 발현하여 *rapC* null 세포의 표현형이 야생형과 비교해보았고 또는 C- 말단이 RapC를 포함하지 않고 RapC에 Ras 도메인만 가지고 RapC에 기능을 하는

지 알아보았다. 온전한 RapC만이 *rapC* null 세포의 결함을 보완할 수 있었다. 그리고 RapC의 C-말단이 RapA에 기능에 어떠한 영향이 있는지 확인하였을 때 RapA는 *rapC* null 세포의 결함을 보완하지 못 하였지만 RapC의 C-말단과 융합된 재조합 RapA는 RapC의 세포 형태, 이동, 발생에서 RapC와 유사한 기능을 하였다.

발생단계는 배 발생과 세포 이동에 중요한 메커니즘이다. 딕티오스텔리움은 모든 음식이 고갈되었을 때부터 발생단계를 거치게 된다. 발생단계가 시작될 때 딕티오스텔리움은 화학 유인물질을 분비하여 서로 소통하여 다세포 응집체를 형성한다. 응집체는 최종적으로 단일 마운드에서 하나의 tip를 형성하게 된다. 이전 연구에서는 RapC가 적절한 발달에 필요했으며 *rapC* null 세포는 단일 마운드 및 다중 분기 발달 구조에서 여러 팁을 형성하는 발달 표현형을 가지고 있음을 보여주었다. RapC가 결실 된 세포의 발달 결함에 대한 이유를 조사하기 위해 cAMP 신호 전달 경로와 막 잠재력이 발달에 관여하는지 확인했다. 카페인은 아데노신의 길항제로 알려져 있으며 진동하는 cAMP 파동을 억제한다. 카페인 존재 하에 야생형 세포가 개발되었을 때, 흥미롭게도 *rapC* null 세포와 유사한 여러 팁을 형성하는 발달 표현형이 관찰했다. 이러한 결과는 cAMP 신호 전달 경로가 발달 표현형을 일으키는 *rapC* null 세포에서 결함이 있을 수 있음을 시사한다. 막 잠재력이 발달에 관여하는지 확인하기 위해 나는 발달 매체에서 다양한 농도의 칼륨에서 발달을 조사했다. 야생형 세포와 비교하여 고농도의 칼륨에서 발달에 큰 차이가 없었다. 그러나 고농도의 칼륨이 존재 상태에서 세포에 전기장을 가하면 다중 팁이 형성되었다. 개발 중 다중 팁 형성 메커니즘과 Ras 신호 전달 경로를 이해하기 위해 추가 연구가 수행되어야 한다.

❖ This part is to be submitted for publication as Dongju Kim and Taeck J. Jeon, 2020.

Part I. Reversible function of RapA with the C-terminal region of RapC in *Dictyostelium*

I. INTRODUCTION

The social amoeba, *Dictyostelium discoideum*, has been used as a model system for investigation of chemotaxis, directional cell movement towards chemoattractants, over the past 40 years. *Dictyostelium* is a free-living soil amoeba that feeds on bacteria. These organisms chase bacteria by chemotaxing towards folic acid, which is secreted by the bacteria (Kortholt and van Haastert, 2008). This process is very similar to that of macrophages or neutrophils chasing bacteria. Upon starvation, *Dictyostelium* undergoes a tightly regulated multicellular developmental process in which they secrete cAMP and move toward cAMP via chemotaxis, leading to the eventual formation of fruiting bodies (Chisholm and Firtel, 2004).

The *Dictyostelium* Ras GTPase subfamily contains 15 proteins, 11 Ras, 3 Rap and one Rheb-related protein. Ras proteins are involved in cancer development. Ras activation is associated with autism and other nervous system disorders(Simanshu et al., 2017). Ras is a small monomeric GTPase that acts as a molecular switch (molecular switch) in the cell signaling (Self et al., 2001). Ras cycles between an inactive GDP-bound and an active GTP-bound conformation. Ras activation or deactivation is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) respectively (Mun et al., 2014). Ras proteins are rapidly activated upon chemoattractant stimulation

downstream from the receptors and heterotrimeric G proteins. The activated Ras proteins are enriched at the leading edge of the chemotaxing cells, where they locally activate the signaling molecules including phosphatidylinositol 3-kinases (PI3Ks). The reciprocal localization and activation of PI3K and PTEN lead to the accumulation of phosphatidylinositol (3,4,5) trispohsphate (PIP3) at the leading edge, which helps guide the local polymerization of F-actin and pseudopod extension possibly by recruiting pleckstrin homology (PH) domain-containing proteins, such as PhdA, CRAC, and PKB (Raaijmakers and Bos, 2009).

Dictyostelium Rap proteins are composed of RapA(Rap1), RapB, and RapC. RapA functions mainly in controlling cell adhesion through dynamic cytoskeleton rearrangements in response to diverse external stimuli, F-actin assembly and myosin disassembly (Hilbi and Kortholt, 2019). RapA is rapidly and transiently activated in response to chemoattractant stimulation at the leading edge of chemotaxing cells and helps establish cell polarity by locally modulating myosin II assembly and disassembly through the RapA/Phg2 signaling pathway (Shin et al., 2010). Spatial and temporal regulation of Rap1 activity by RapA GAPs is required for proper cell migration as well as cell differentiation and development (Jeon et al., 2007a). RapA has also been linked to the control of F-actin polymerization. In mammalian cells, RapA promotes cell spreading by binding to and localizing the RacGEFs Vav2 and Tiam1 to the sites of active lamellipodia extension (Arthur et al., 2004). In *Dictyostelium*, F-actin assembly was increased in the cells expressing constitutively active RapA (Jeon et al., 2007b). The functions of RapB in *Dictyostelium* have not been examined. RapC is required for controlling cell adhesion, likely by inhibiting strong cell-substrate adhesion. RapC seems to play a negative role in cell spreading, as loss of RapC leads to cell spreading, and is required for proper cytokinesis. Also, RapC is required for the control of migration speed possibly by negatively regulating cell-substrate adhesion rather than the direction of

migration or directional sensing during chemotaxis. RapC seems to play an important role in the tip formation from the aggregates at the late mound stage of development. Loss of RapC resulted in aberrations in multicellular development after the normal aggregation stage, producing multi-tipped mounds and multi-branched developmental structures. These multi-tip-forming phenotypes appear to be unique to *rapC* null cells, in contrast to the other Rap protein previously described. RapC has an additional stretch of residues at the C-terminus, unlike other Rap proteins. RapC plays an opposite function to RapA in cell migration and cell adhesion. RapA and RapC appear to play antagonistic roles in cell adhesion and cell migration. The functions of RapC are very different from those of RapA in the control of cell spreading, cell adhesion and migration, and multicellular development, despite its high sequence homology with RapA (Park et al., 2018a). I examined whether the C-terminus of RapC plays a role in reversing the functions of RapA using a recombinant RapA fused with the C-terminus of RapC.

II. MATERIALS AND METHODS

II-1. Strains and cell culture

The *D. discoideum* KAx-3 strain was axenically cultured in HL5 medium at 22 °C. Cells expressing RapA, RapC and other mutated proteins were maintained with 10 μg/mL G418. *rapC* null cells (Park et al., 2018b) were cultured in the presence of 10 μg/mL of bsr.

II-2. Plasmids

The coding sequence of *rapC* and *rapA* was obtained by reverse transcription–polymerase chain reaction (RT–PCR) with primers. The amplification fragment was ligated into the *Eco*RI–*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. The RapC C-terminal deletion of the RapC sequence was also constructed by PCR using the full-length *rapC* cDNA coding sequence as the template and as the primers. All clones were confirmed by DNA sequencing. The amplified fragments were ligated into the *Eco*RI–*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. The Gibson Assembly cloning kit was used to clone RapA fused with C-terminus of RapC(RapA-tail). To generate RapA-tail, RapA and C-terminal of RapC were amplified by PCR using Gibson assembly Kit (Gibson et al., 2010) (Table 1) and cloned into the *Eco*RI/ *Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment.

Table 1. PCR primers used in this study.

Name	Forward primer (5`→3`)	Reverse primer (5`→3`)
RapC	AAAATGCAAACTATAAAG	CAAATTAGTACATTTATCAA
RapCΔtail	AAAATGCAAACCTATAAAG	TTTCTATTTTTTCTATTTTTTCTTATTG
RapA-tail	ATGCCTCTTAGAGAATTTAAAATCG	ACGAAATAACTTTCTTTTCTTTTGA
	AAAGAAAAGAAAACATCATAAAAAG	CAAATTAGTACATTTATCAA

II-3. Cell adhesion assay

Log-phase growing cells on plates were washed with 12 mM Na/K phosphate buffer (pH 6.1) and resuspended at the density of 2×10^6 cells/mL. Cells (4×10^5 in 200 μL) were plated and attached on the 6-well culture dishes. Before shaking the plates, cells were photographed and counted for calculating the total cell number. The plates were constantly shaken at 150 rpm for 1 h to detach the cells from the plates, and the attached cells were photographed and counted (attached cells) after the medium containing the detached cells was removed. Cell adhesion was shown as a percentage of attached cells compared with total cells.

II-4. Development assay

Development assay was performed as described previously (Jeon et al., 2009). Exponentially growing cells were washed twice with 12 mM Na/K phosphate buffer (pH 6.1) and resuspended at a density of 3.5×10^7 cells/mL and then 50 μL of the cells were dropped on Na/K phosphate agar plates and developed for 24 h. The multicellular developmental morphology of the cells was examined under a phase-contrast microscope.

II-5. Chemotaxis assay

Chemotaxis of the cells toward cAMP chemoattractants, was examined as described previously (Jeon et al., 2007a). Aggregation-competent cells were prepared by incubating cells at a density of 5×10^6 cells/mL in 12 mM Na/K phosphate buffer (pH 6.1) for 10 h with a constant shaking (110 rpm). Cell migration was analyzed using a Dunn Chemotaxis Chamber (SVDCC100, UK). The images of chemotaxing cells were taken at time-lapse intervals of 1 min for 1 h. The data were analyzed using the NIS-Elements software (Tokyo, Japan, Nikon) and Image J software (National Institutes of Health).

II-6. DAPI staining

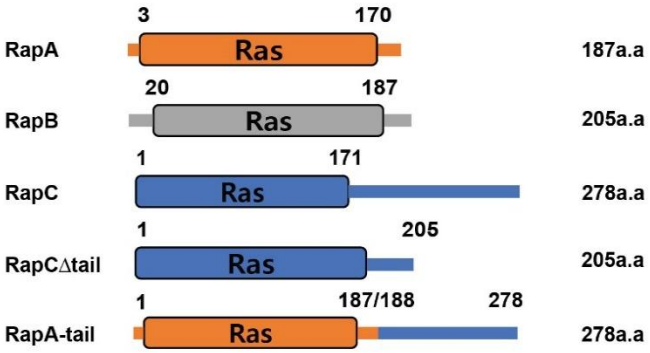
Exponentially growing cells were placed on the coverslip and then fixed with 3.7 % formaldehyde for 15 min. The fixed cells were washed with phosphate-buffered saline (PBS buffer, pH 7.4), permeabilized with 0.1% Triton X-100 for 1 min, and then stained with 0.5 μg/mL of Hoechst dye in 1mL of mounting solution Fluoromount-G. Images were captured using NIS-elements software (Tokyo, Japan, Nikon).

III. RESULTS

III-1. Characterization of the gene encoding RapC and RapA.

To further characterize the Rap proteins, I performed computer-based analyses. *Dictyostelium* RapA (DDB_G0291237) is composed of 187 amino acids (expected molecular mass 21 kDa) and contains a RAS domain at the N-terminal region. RapB (DDB_G0272857) is composed of 205 amino acids (expected molecular mass 23 kDa) and contains a RAS domain at the N-terminal region. *Dictyostelium* RapC (DDB_G0270340) is composed of 278 amino acids (expected molecular mass 31 kDa) and contains a RAS domain at the N-terminal region. RapC has a long C-terminus unlike other Rap proteins. To determine if the C-terminus of RapC plays a role in the regulation of cellular processes by RapC, I prepared expression plasmids for expressing recombinant RapC with a full-length RapC and a truncated RapC lacking its tail region. Next, I prepared recombinant RapA fused with C- terminal region of RapC (Fig 1A).

A



B

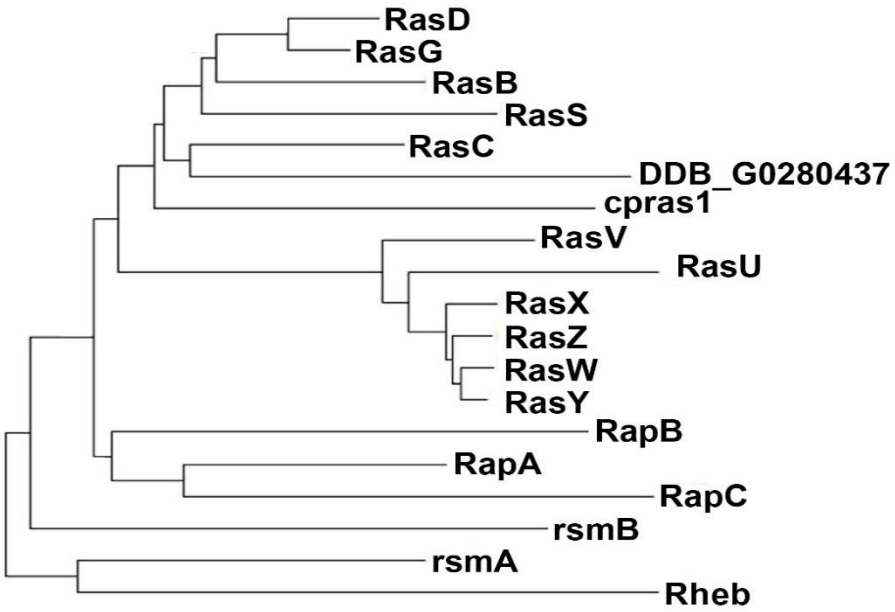


Fig 1. Domain structure of Rap proteins and phylogenetic tree of Ras domains.

(A) Domain structure of RapA, RapB, and RapC. (B) Phylogenetic tree analysis of the Ras domain from *Dictyostelium*. DRasD, *Dictyostelium discoideum* (dictyBaseID: DDBG0292996); DRasG , *Dictyostelium discoideum* (dictyBaseID: DDBG0293434); DRasB, *Dictyostelium discoideum* (dictyBaseID: DDBG0292998); DRasS, *Dictyostelium discoideum* (dictyBaseID: DDBG0283537); DRasC, *Dictyostelium discoideum* (dictyBaseID: DDBG0281385); DDB_G0280437, *Dictyostelium discoideum* (dictyBaseID: DDBG0280437); Dcpras1, *Dictyostelium discoideum* (dictyBaseID: DDBG0277381); DRasV, *Dictyostelium discoideum* (dictyBaseID: DDBG0270736);DRasU, *Dictyostelium discoideum* (dictyBaseID: DDBG0270138); DRasX, *Dictyostelium discoideum* (dictyBaseID: DDBG0270124); DRasZ, *Dictyostelium discoideum* (dictyBaseID: DDBG0270140); DRasW, *Dictyostelium discoideum* (dictyBaseID: DDBG0270122); DRasY, *Dictyostelium discoideum* (dictyBaseID: DDBG0270126); DRapB, *Dictyostelium discoideum* (dictyBaseID: DDBG0272857); DRapA, *Dictyostelium discoideum* (dictyBaseID: DDBG0291237); DRapC, *Dictyostelium discoideum* (dictyBaseID: DDBG0270340); DrsmB, *Dictyostelium discoideum* (dictyBaseID: DDBG0281253); DrsmA, *Dictyostelium discoideum* (dictyBaseID: DDBG0283547); DRheb, *Dictyostelium discoideum* (dictyBaseID: DDBG0277041); these sequences are available at www.dictybase.org.

III-2. The C-terminus of RapC is required for the regulation of cell morphology.

To determine if the C-terminus of RapC plays a role in the regulation of cellular processes by RapC, I prepared expression plasmids for expressing green fluorescent proteins (GFP)-fused recombinant RapC with a full-length RapC and a truncated RapC lacking its tail region (RapCΔtail). RapCΔtail was created by cloning only Ras domain in RapC (Fig 2A). These plasmids were introduced into *rapC* null cells. To examine the roles of RapCΔtail in controlling cell morphology, I compared the morphological characteristics of *rapC* null cells expressing a full-length RapC and RapCΔtail with those of wild-type cells, and *rapC* null cells. I measured the size of the cells using NIS-element software (Fig 2). *rapC* null cells are spread as reported previously (Park et al., 2018b). The enlarged morphology of *rapC* null cells were complemented by wild-type RapC but not RapC missing its tail C-terminal region. Quantification of the cell size showed that the mean size of *rapC* null cells and *rapC* null cells expressing RapCΔtail was approximately 2.5-fold larger than that of wild-type cells (Fig 2C and 2D). These results indicate that the C-terminus of RapC is essential for the regulation of cell morphology by RapC.

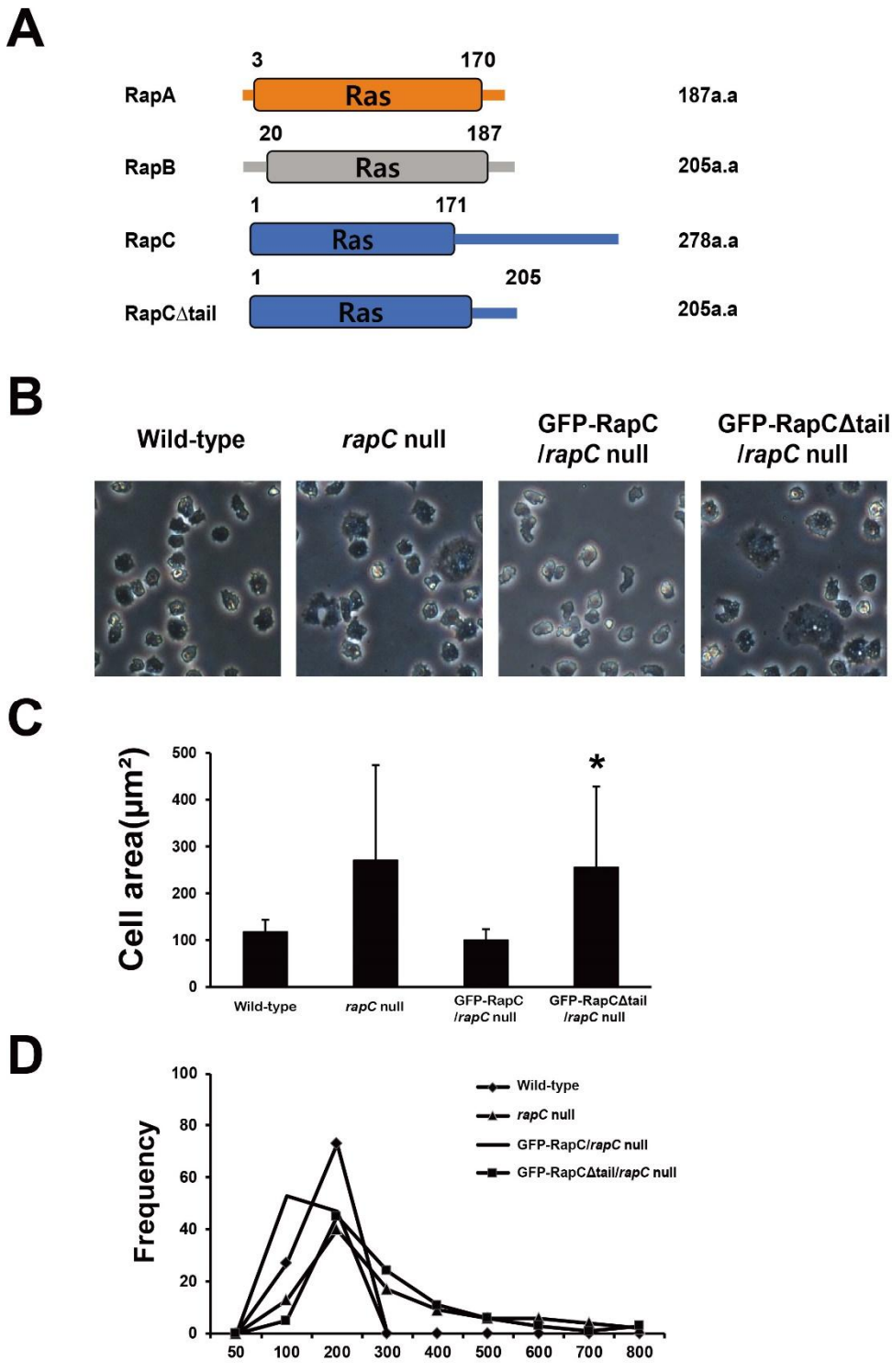


Fig 2. The spread morphology of *rapC* null cells was not rescued by GFP-RapCΔtail.

(A) RapC missing the C-terminus (RapCΔtail) are shown. These proteins were expressed in *rapC* null cells as GFP-fusion protein. (B) Morphology of the vegetative cells. KAx3, *rapC* null, GFP-RapC/*rapC* null, and GFP-RapCΔtail/*rapC* null were photographed. (C) Measurement of cell areas of cell using NIS-element software. Error bars represent ± SD of three independent experiments (*p<0.05 compared to the control). (D) Frequency of the cell size is shown.

III-3 The C-terminus of RapC is required for the regulation of cell adhesion and cytokinesis.

To determine if the phenotypes of *rapC* null cells in cell adhesion is rescued by RapCΔtail, I measured the strength of cell adhesion by counting the number of the attached cells after shaking and washing off the detached cells (Fig 3). *rapC* null cells exhibited strong adhesion compared to wild-type cells. The phenotypes of *rapC* null cells in cell adhesion was recovered by full-length RapC, but not by RapC missing the C-terminus (RapCΔtail). This result suggests that the C-terminus of RapC is essential for the regulation of cell adhesion by RapC. RapC appears to play a negative role for proper cytokinesis (Park et al., 2018b). To determine if the C-terminus of RapC is required for cytokinesis, I examined the cytokinesis of *rapC* null cells expressing full-length RapC and RapCΔtail by counting the number of nuclei (Fig 3). Most wild-type cells contained one or two nuclei, whereas *rapC* null cells had two nuclei and some cells had more than eight nuclei. These phenotypes of *rapC* null cells in cytokinesis was rescued by full-length RapC but not RapCΔtail, suggesting that the C-terminus is essential for cytokinesis.

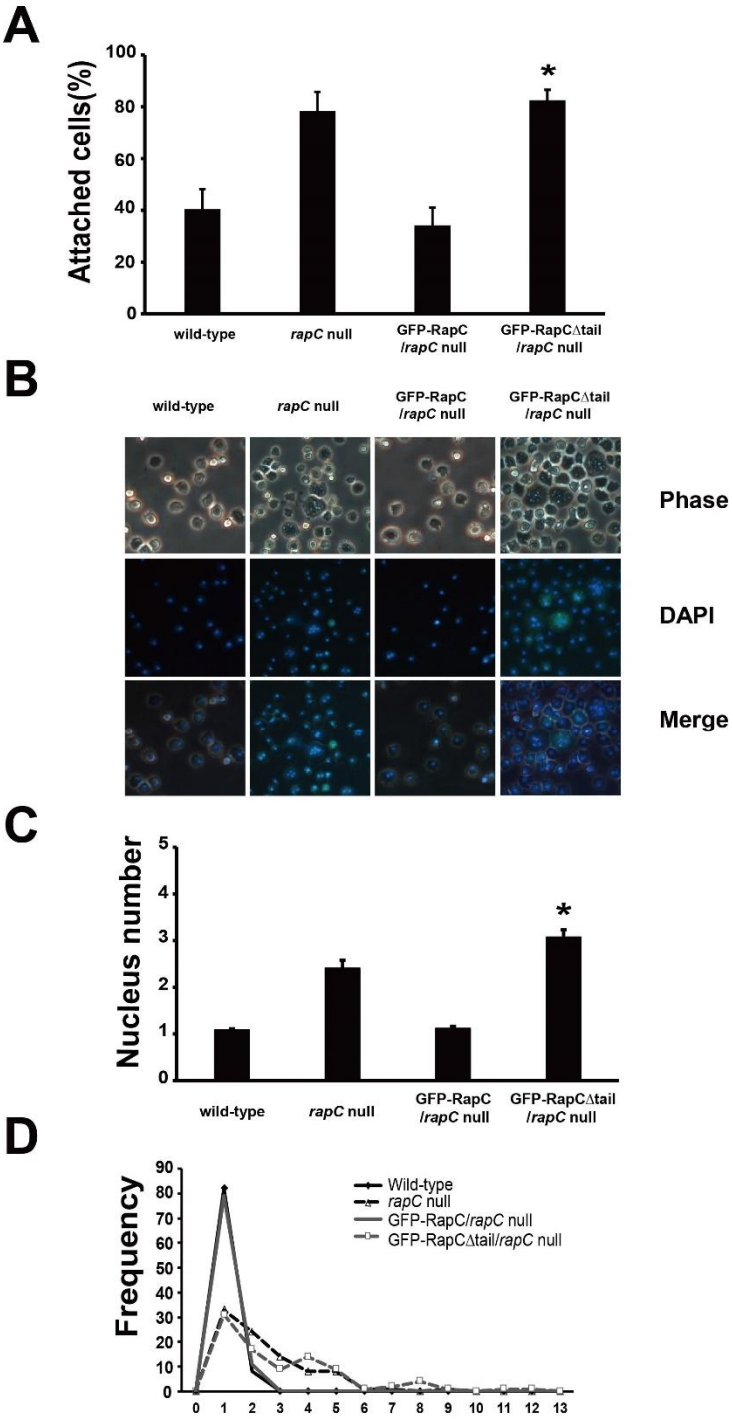


Fig 3. Cell adhesion and cytokinesis of *rapC* null cells by RapCΔtail.

(A) Cell-substrate adhesion. Adhesion of the cells to the substrate was expressed as a percentage of attached cells to total cells. Error bars represent SD of three independent experiments (*p<0.05 compared to the control). (B) Representative DAPI images of the cells. Exponentially growing wild-type, *rapC* null cells, and *rapC* null cells expressing GFP-RapC or GFP-RapCΔtail. Corresponding phase-contrast and merged images are shown on the top and bottom panels, respectively. (C) Quantification of the number of nuclei in the cells. Data represent the means ± SD of three independent experiments (*p<0.05 compared to the control). (D) Frequency of the number of nuclei of the cells.

III-4. The C-terminus of RapC is required for the regulation of development and cell motility.

To examine the possible roles of the C-teriminus of RapC in development, I performed a developmental assay. The previous data showed *rapC* null cells had multiple fruiting bodies (Park et al., 2018b). These phenotypes were rescued by full-length RapC but not by tail-deleted RapC, suggesting that the C-terminus is required for the full activity of RapC during development (Fig 4).

rapC null cells have defects in the control of migration speed in chemoattractant–directed cell migration. To investigate whether RapCΔtail were able to rescue the phenotypes of *rapC* null cells in cAMP-mediated chemotaxis, I examined cell motility of the cells using a Dunn chemotaxis chamber (Fig 5). Aggregation – competent cells were prepared by starving the cells in Na/K phosphate buffer for 10 h and subjected to the chemotaxis assay. In directionality, which is the index showing how straight the cells move, all of the cells including wild-type, *rapC* null cells, GFP-RapC cells, and GFP-RapCΔtail cells showed no significant difference (Fig 5B). Wild-type cells moved at 8.0 μm/min toward the cAMP source. In contrast, *rapC* null cells showed low moving speed with 4.0 μm/min and GFP-RapC cells had had migration speed similar to that of wild-type cells. RapCΔtail cells showed low moving speed with 2.0 μm/min (Fig 5C). Similar to the results of cell spreading and adhesion, the slow migration speed of *rapC* null cells in chemotaxis was recovered by full-length RapC but not by tail-deleted RapC, suggesting that the C-terminus is required for the regulation of cell migration by RapC.

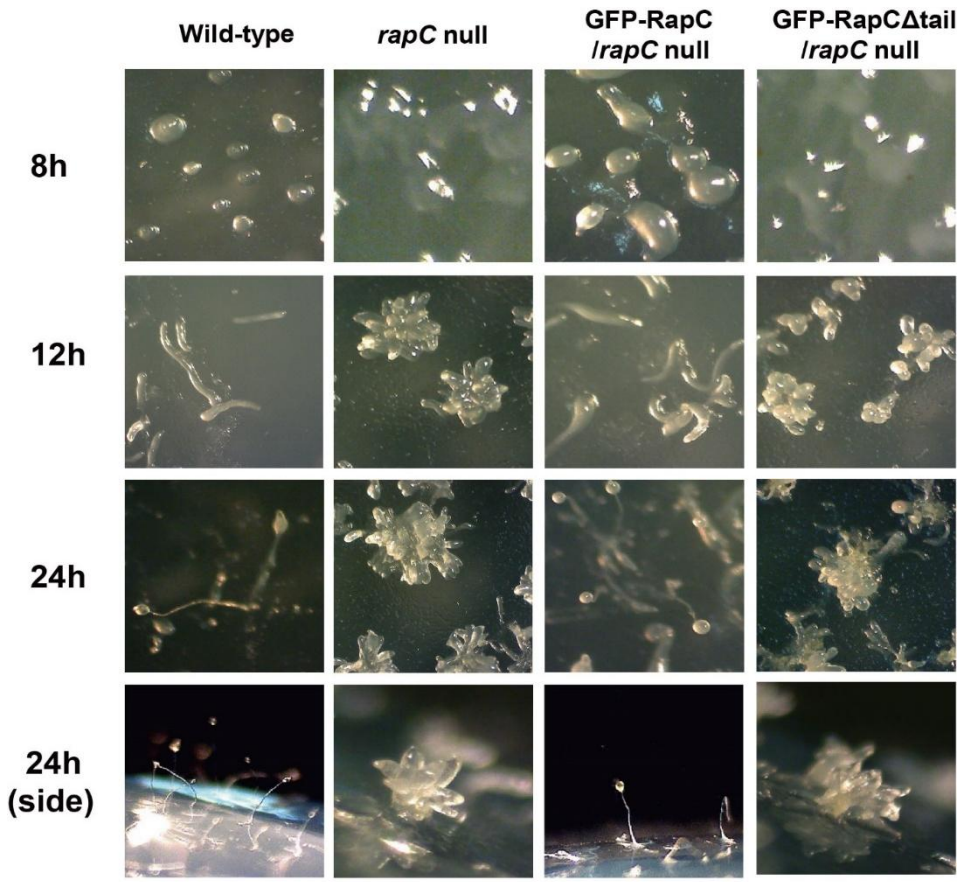
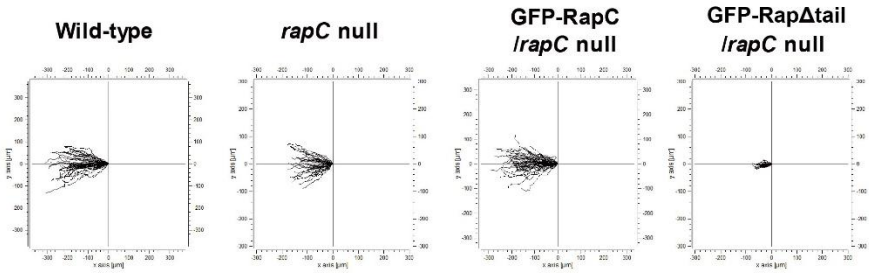


Fig 4. Development of *rapC* null cells by RapCΔtail.

Development on non-nutrient (Na/K) 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 8 h (aggregation stage), at 12 h (tip-formation and slug stage), and at 24 h (fruiting body formation stage) are shown. Side view of developmental structures at 24 h are shown at the bottom.

A



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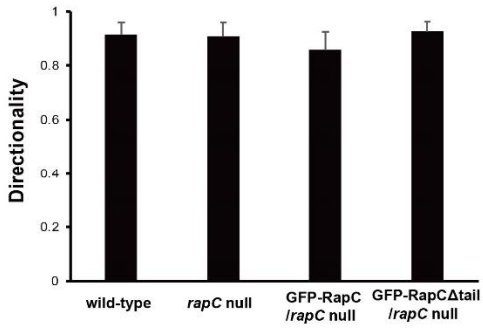

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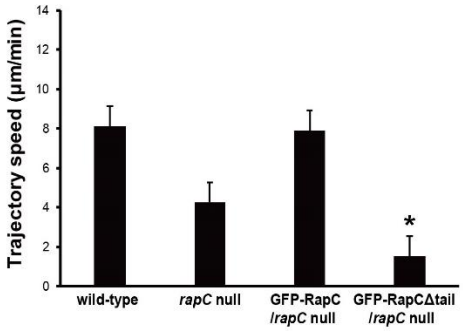

Fig 5. Cell motility of *rapC* null cells by RapCΔtail.

(A) Trajectories of cell movements towards cAMP in Dunn chemotaxis chamber. Aggregation competent cells were placed in a Dunn chemotaxis chamber, and the migration of the cells towards a high gradient of cAMP chemoattractants were analyzed. (B) Directionality of the cells in chemotaxis. Directionality measures how straightly cells move. The direction of cell moving in a straight line is 1. (C) Trajectory speed is the total distance traveled by the cell divided by the time. These experiments were performed at least three times. Error bar indicated SD (*$p<0.05$ compared to the control by the t-test).

III-5. Localization of RapC.

I examined the subcellular localization of RapC. GFP-RapC and RapCΔtail were observed on the plasma membrane and also found in the intracellular membranes (Fig 6). To test if GFP-RapC and RapCΔtail are completely expressed, I examined the size of the expressed proteins by immunoblotting using anti-antibodies against GFP. GFP-RapC and GFP-RapCΔtail were detected at bands of 55 kDa and 50 kDa, respectively (Fig 6).

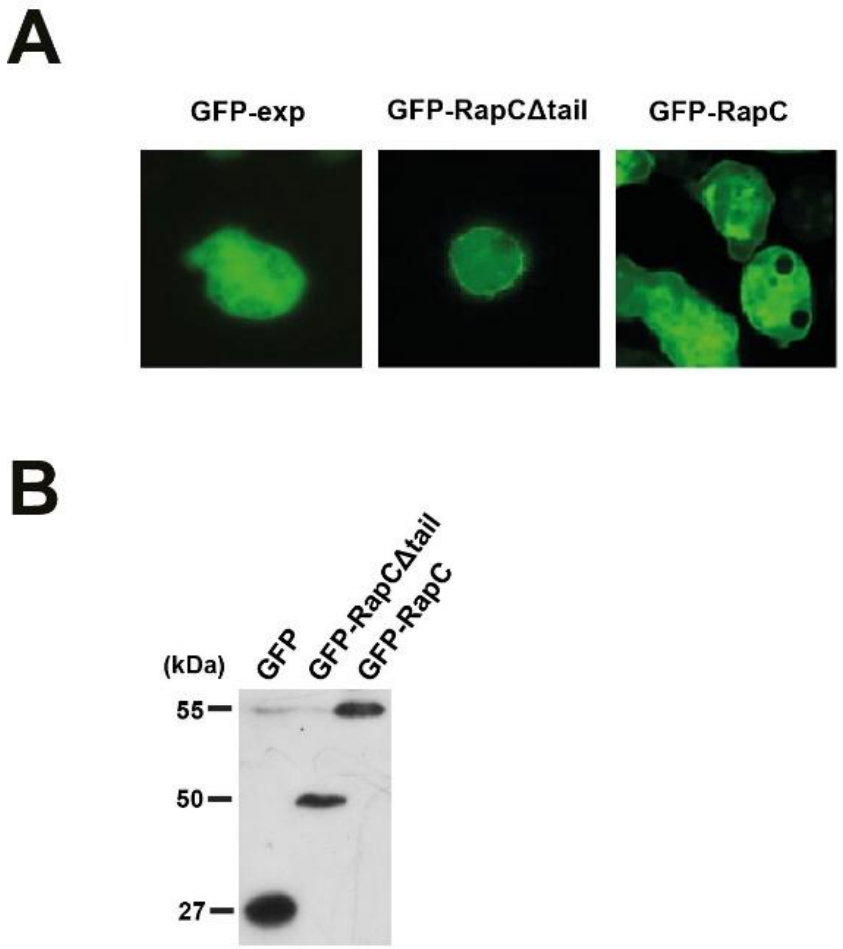

Fig 6. Localization of GFP-RapC and GFP-RapCΔtail

(A) Subcellular localization of GFP-exp, GFP-RapCΔtail and GFP-RapC. (B) Western blotting for GFP-RapC and GFP-RapCΔtail. Proteins were extracted from *rapC* null cells expressing GFP-RapC and GFP-RapCΔtail and then subjected to immunoblotting analysis using GFP anti-antibodies (expected size of GFP-RapC, 55 kDa, and RapCΔtail, 50 kDa).

III-6. The C-terminus of RapC reverses the function of RapA in cell morphology.

To determine if the functions of RapA can be changed by the C-terminus of RapC, I prepared *rapC* null cells expressing RapA or the recombinant RapA fused with the C-terminus of RapC and examined whether the phenotypes were recovered. To determine the roles of RapA-tail in controlling cell morphology, I compared the morphological characteristics of RapA cells and RapA-tail cells with those of wild-type cells and *rapC* null cells (Fig 6). RapA/*rapC* null cells had the same cell morphology as *rapC* null cells, which are spread and enlarged. The phenotypes of *rapC* null cells were completely recovered by not only full-length RapC but also a recombinant RapA fused with the C-terminus of RapC. RapA-tail/*rapC* null cells showed almost same cell morphology as wild-type cells. Quantification of the cell size showed that the mean size of RapA/*rapC* null cells was approximately 2.5-fold larger than that of wild-type cells (Fig 6C and 6D). The mean size of RapA-tail/*rapC* null cells was the same as wild-type cells. These results suggest that the functions of RapA in controlling cell morphology were changed to the opposite by the C-terminus of RapC.

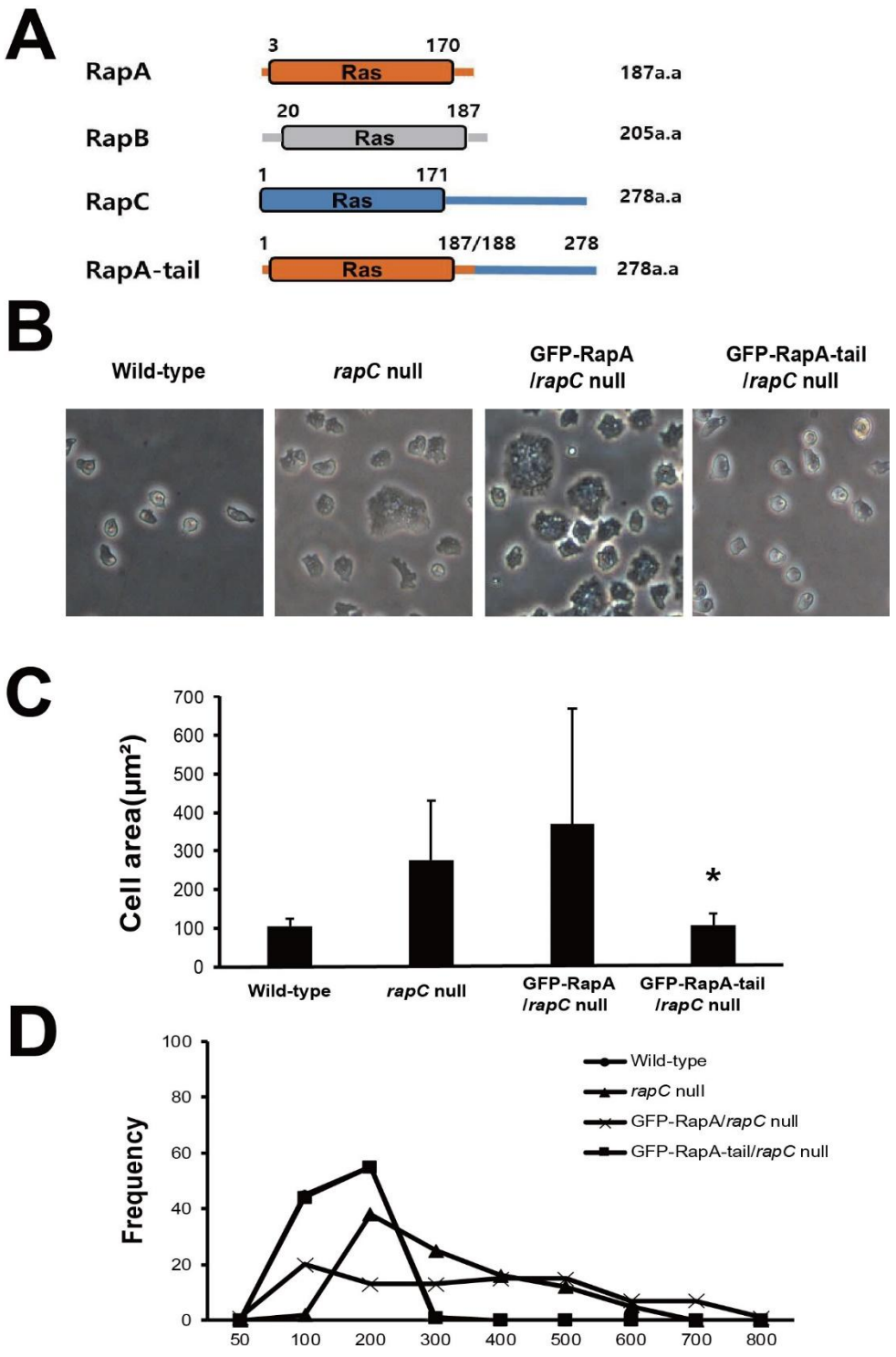

Fig 7. The spread morphology of *rapC* null cells was rescued by GFP-RapA-tail.

(A) Diagram of a recombinant RapA fused with the C-terminus of RapC (GFP-RapA-tail). (B) Cell morphology of the cells. Wild-type, *rapC null*, GFP-RapA/*rapC* null, and GFP-RapA-tail/*rapC* null cells were photographed. (C) Measurement of cell areas of the cells using NIS-element software. Error bars represent SD of three independent experiments (*p<0.05 compared to the control). (D) Frequency of the cell size is shown.

III-7. The C-terminus of RapC reverses the function of RapA in cell adhesion and cytokinesis.

RapA is a key regulator of cell adhesion and cytoskeleton rearrangement. The increased cell adhesion of cells lacking RapC are similar to those of cells expressing constitutively active RapA (Rap1). The phenotypes of *rapC* null cells in cell adhesion was completely recovered by RapA-tail, suggesting RapA-tail has the function similar to RapC in cell adhesion (Fig 8). To investigate roles of RapA and RapC in cytokinesis, I compared the number of nuclei in the cells by DAPI staining (Fig 8B, 8C, and 8D). *rapC* null cells contained approximately 3 nuclei while wild-type cells had one of the average number of nuclei, indicating that RapC is required for proper cytokinesis. The defects of *rapC* null cells in cytokinesis were rescued by RapA-tail but not by RapA. These results suggest that the C-terminus of RapC changed the functions of RapA to those similar to RapC.

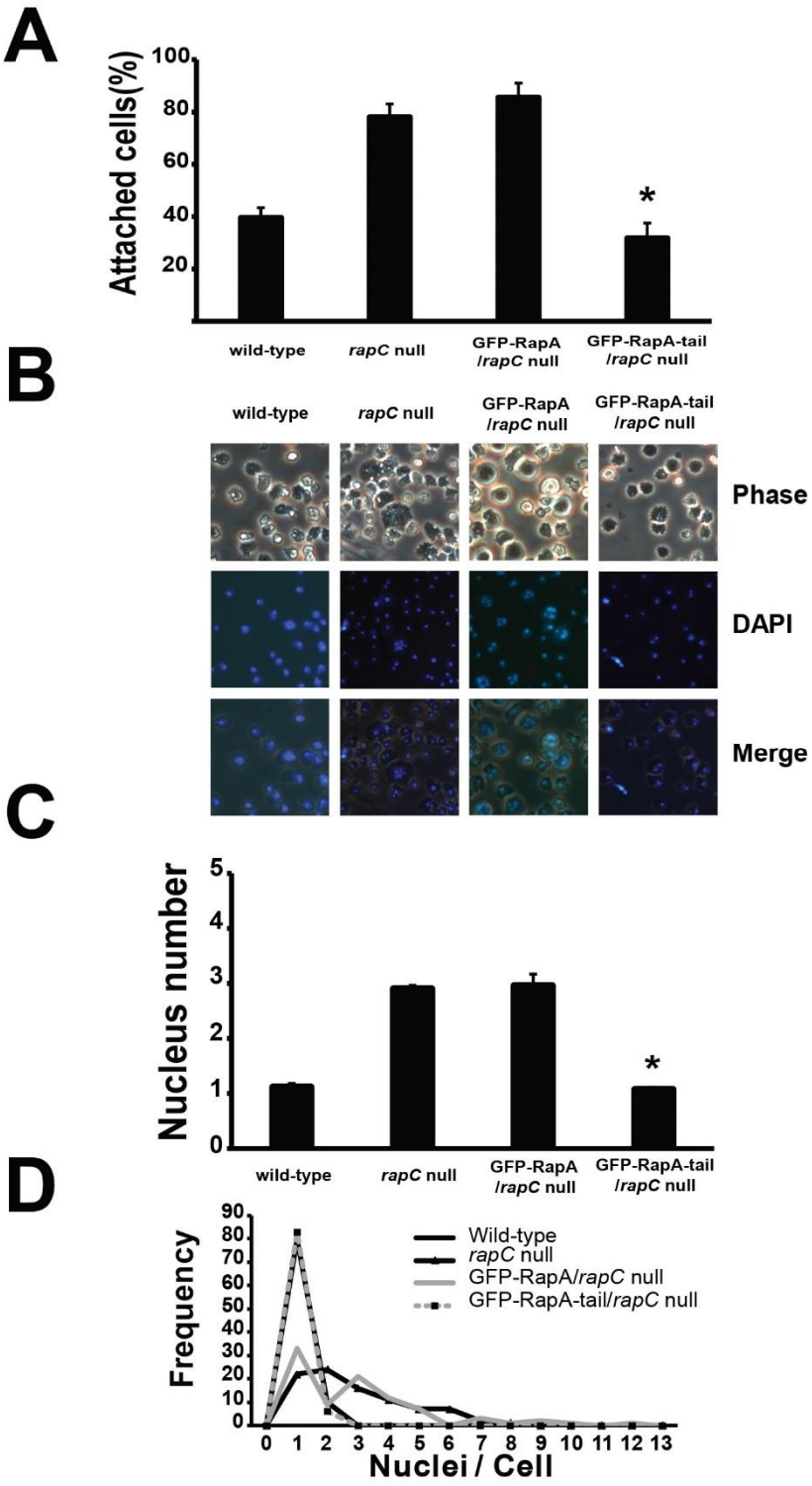

Fig 8. Cell adhesion and cytokinesis of *rapC* null cells by RapA-tail.

(A) Cell-substrate adhesion. Adhesion of the cells to the substrate was expressed as a percentage of attached cells to total cells. (B) Representative DAPI images of the cells. Exponentially growing cells including wild-type cells, *rapC* null cells, *rapC* null cells expressing GFP-RapA, and *rapC* null cells expressing GFP-RapA-tail were stained with Hoechst dye. Corresponding phase-contrast and merged images are shown on the top and bottom panels, respectively. (C) Quantification of the number of nuclei in the cells. Data are mean values Error bars ± SD from three independent experiments (*$p<0.05$ compared to the control by the t-test). (D) Frequency of the number of nuclei of the cells.

III-8. The C-terminus of RapC reverses the function of RapA in development and cell migration.

RapC has been reported to be involved in multicellular development. To examine the possible roles of RapA-tail in development, I performed a developmental assay. The previous data showed *rapC* null cells aggregated to form multiple tips from a single mound and finally forming multiple fruiting bodies (Fig 9). The multi-tip formation of *rapC* null cells were complemented by RapA-tail but not by RapA. These results indicate that RapA-tail has a function similar to RapC in development. *rapC* null cells have defects in chemoattractant–directed cell migration. To investigate whether RapA-tail functions as a RapC in cAMP-mediated chemotaxis, a chemotaxis assay was performed with wild-type cells, *rapC* null cells, *rapC* null cells expressing GFP-RapA, and *rapC* null cells expressing GFP-RapA-tail using a Dunn chemotaxis chamber (Fig 10). Aggregation –competent cells were prepared by starving the cells in Na/K phosphate buffer for 10 h and subjected to chemotaxis assay (Fig 10). There was no significant difference in directionality of the chemotaxing cells. However, *rapC* null cells showed decreased migration speed as previously described (Park et al., 2018b). Wild-type cells moved at 8.0 μm/min toward the cAMP source. In contrast, *rapC* null cells showed low moving speed with 2.0 μm/min. GFP-RapA cells showed migration speed, 1.8 um/min, similar to *rapC* null cells. RapA-tail cells recovered the migration speed with 8.0 μm/min (Fig 10C). These results indicate that RapA-tail recovered the decreased migration speed of *rapC* null cells and the C-terminus modified the functions of RapA to become similar to those of RapC in cell migration.

I examined the subcellular localization of GFP-RapA and RapA-tail. GFP-RapA-tail was observed on the plasma membrane and also found on the intracellular membranes (Fig 11). Immunoblotting analysis using anti-GFP antibodies confirmed that GFP-RapA and GFP-RapA-tail were expressed in *rapC* null cells. The expected sizes of GFP-RapA and GFP-RapA-tail was 48 kD and 55 kD, respectively, and protein bands corresponding to the expected sizes were detected (Fig 11).

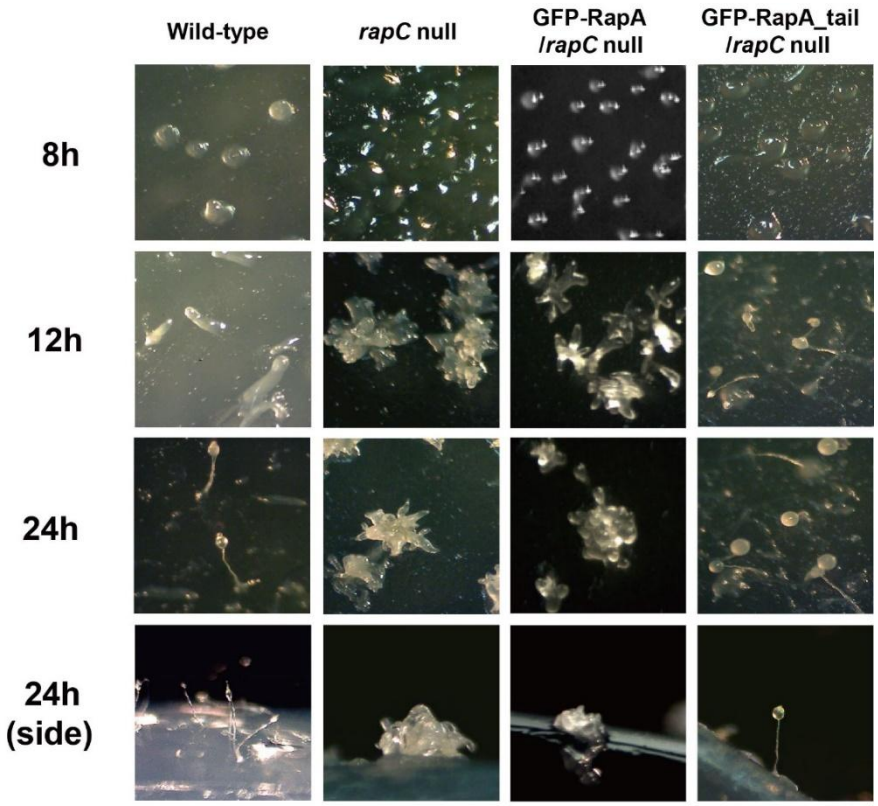

Fig 9. Development of *rapC* null cells by RapA-tail.

Development of the cells on non-nutrient agar plates. 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 8 h (aggregation stage), at 12 h (tip-formation and slug stage), and at 24 h (fruiting body formation stage) are shown. Side view of developmental structures at 24 h are shown at the bottom.

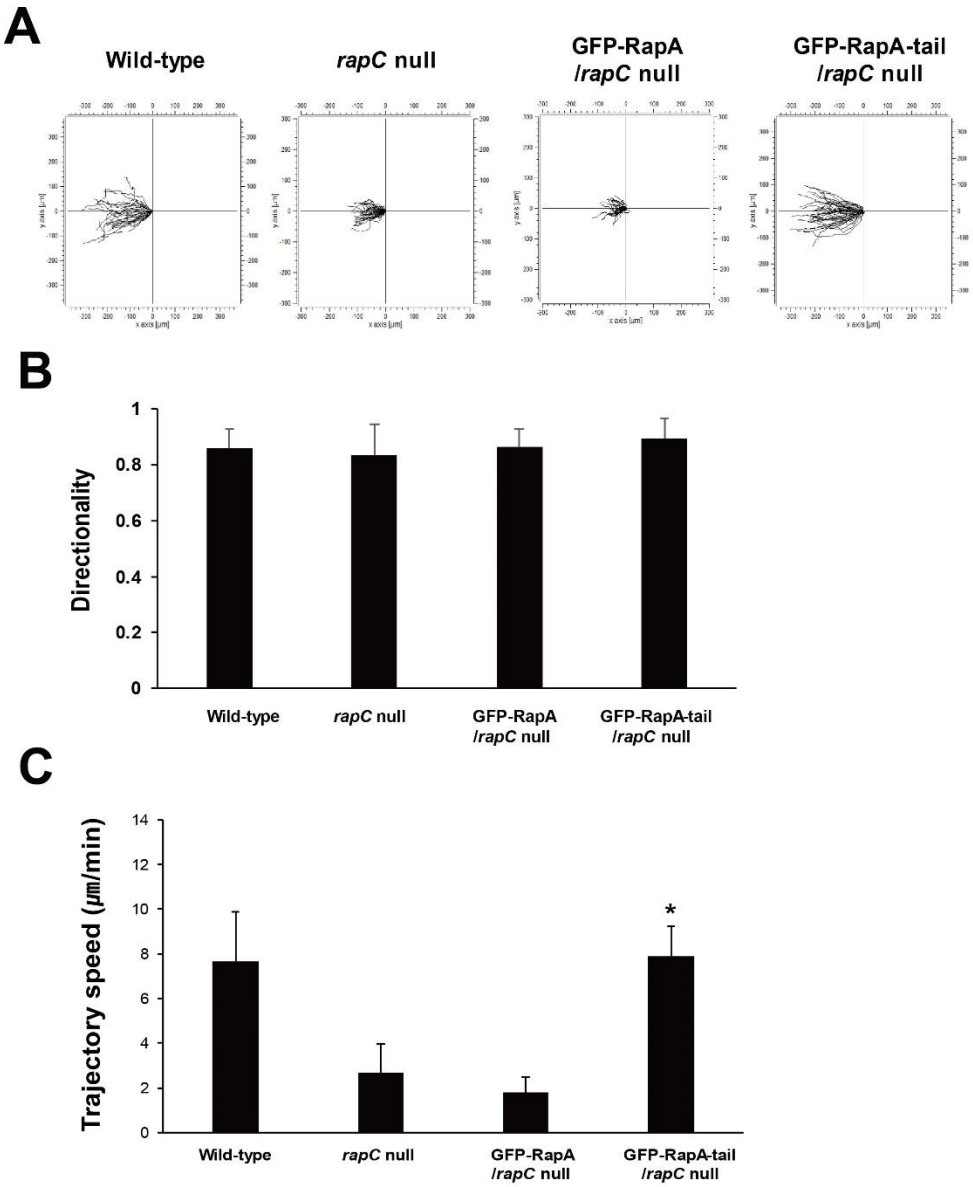

Fig 10. Cell motility of *rapC* null cells by RapA-tail.

(A) Trajectories of the chemotaxing cells. Aggregation competent wild-type cells, *rapC* null cells, GFP-RapA/*rapC* null cells, and GFP-RapA-tail/*rapC* null cells were placed in a Dunn chemotaxis chamber. The migration of the cells was recorded for 1 h with intervals of 1 min. Each line represents the track of a single cell chemotaxing towards cAMP. (B) Directionality of the chemotaxis cells. Directionality measures how straightly cells move. The direction of cell moving in a straight line is 1. (C) Trajectory speed of the chemotaxing cells. Trajectory speed is the total distance traveled by the cell divided by the time. These experiments were performed at least three times. Error bar indicated SD (*$p<0.05$ compared to the control by the t-test).

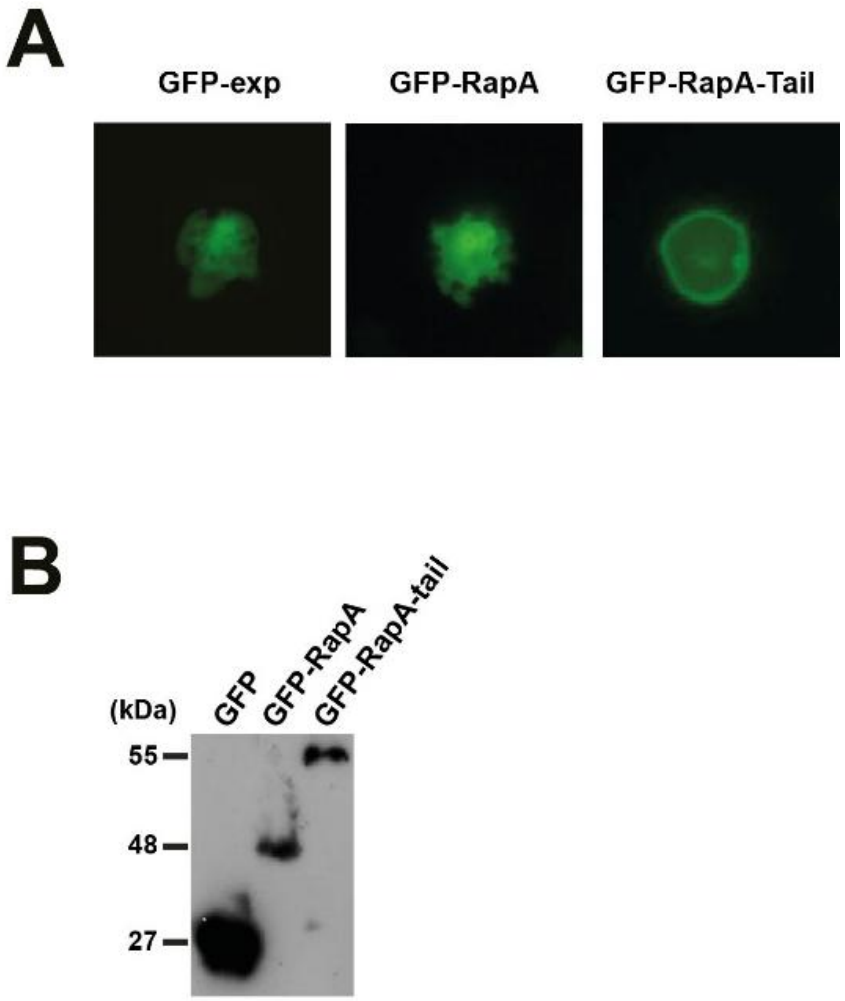

Fig 11. Localization of GFP-RapA.

(A) Localization of GFP, GFP-RapA, and GFP-RapA-tail in living cells. Fluorescent images of cells expressing GFP, GFP-RapA, and GFP-RapA-tail were photographed. (B) Immunoblotting of GFP-RapA and GFP-RapA-tail. Proteins were extracted from cells expressing GFP-RapA and GFP-RapA-tail and subjected to immunoblotting analysis using anti-GFP antibodies.

IV. DISCUSSION

The small GTPase RapA is involved in the control of diverse cellular processes, including integrin-mediated cell adhesion, cadherin-based cell-cell adhesions, cell polarity formation, and cell migration (Jeon et al., 2007b). RapC is a Ras subfamily protein showing the high homology with RapA, which is a key regulator in cell migration and cell adhesion. RapC plays a negative role in controlling cell spreading and cell adhesion, and is required for proper multicellular development. Our results show that the C-terminus of RapC is essential for the regulation of cell morphology, adhesion, cytokinesis, cell migration and development by RapC. RapA is known to function in the ways opposite to RapC in cellular processes. In this study, I tested whether the C-terminus of RapC is able to modify the functions of RapA to become similar to RapC. The results revealed that the phenotypes of *rapC* null cells in cell spread, adhesion, cytokinesis, migration, and development were completely recovered by RapA fused with the C-terminal residues of RapC, indicating that the functions of RapA were reversed to those of RapC by the C-terminus. The mechanisms for the reversal of the functions of RapA by RapC tail should be further investigated. There are two poly-serine domains in the C-terminal region of RapC. The two poly-serine domains are expected to play an important role for functions of RapC opposite to RapA.

RapC seem to play a negative role in cell spreading and is required for proper cytokinesis, as loss of RapC leads to cell spreading and multinucleation. RapA plays a positive role in cell spreading. RapA-tail plays a negative role in cell spreading. RapCΔtail and RapA were unable to reduce the increased size on *rapC* null cells. RapA-tail complemented the phenotypes of *rapC* null cells in cell spreading. These results suggest that RapA-tail plays a similar role to RapC in controlling cell spreading and the C-terminus changes the functions of RapA oppositely to those of RapC.

RapC seem to play an important role in the tip formation from the aggregates at the late mound stage of development. Loss of RapC resulted in aberrations in multicellular development after the normal aggregation stage, producing multi-tipped mounds and multi-branched developmental structures. These multi-tip-forming phenotypes appear to be unique to *rapC* null cells, in contrast to the other phenotypes previously described (Park et al., 2018a). The present study showed that the C-terminus of RapC is essential for recovery of the defective phenotypes of *rapC* null cells during development by RapC. In addition, the results showed that the C-terminus was able to modify the functions of RapA to become similar to RapC in development since the multi-tip formation of *rapC* null cells were completely rescued by RapA fused with the C-terminus of RapC during multicellular development.

RapC is essential for the control of migration speed possibly by negatively regulating cell-substrate adhesion rather than the direction of migration or directional sensing during chemotaxis. RapA and RapC appear to play antagonistic roles in cell migration and cell adhesion (Jeon et al., 2007a; Park et al., 2018b). RapA promotes cell adhesion possibly to decrease the migration speed without affecting directionality in chemotaxis, whereas RapC reduces cell adhesion to increase the migration speed, opposite to those by RapA. The data reveals that the C-terminal region of RapC is able to reverse the functions of RapA, raising the possibility that the C-terminal residues of RapC might be exploited as a suppressor in the Ras oncogenic signaling.

Part II. Multiple tip formation in the development of *rapC* null cells in *Dictyostelium*.

I. INTRODUCTION

Dictyostelium on bacteria until all the food is exhausted. Starvation induces multicellular development; single living cells secrete cAMP to communicate with each other enabling them to form a multicellular aggregate. The aggregates transform into a motile slug which later culminates into a fruiting body with a dead stalk holding a mass of dormant spores (Jaiswal et al., 2012). cAMP, adenosine, ammonia (NH3), and differentiation-inducing factor (DIF) coordinate and regulate cell fate and cell type proportions while occurring in slime molds (Islam et al., 2019). Caffeine is known to induce formation of multiple tips during development(Islam et al., 2019), and *rapC* null cells also form multiple tips (Jaiswal et al., 2012; Park et al., 2018b). Tip dominance is one of the crucial steps in slug volume regulation during morphogenesis in cellular slime molds. The single slug tip, like an organizer of the metazoan embryo, regulates multicellular development (Rubin and Robertson, 1975). The tip of the slug acts as a pacemaker and secretes cAMP signals periodically with a spread speed of 200 μM/min. cAMP plays key roles during *Dictyostelium* development through regulating protein kinase A (PKA) and PKB (Meili et al., 2000). cAMP not only guides cellular aggregation as a chemoattractant to form the signaling center but also functions to regulate prespore differentiation (Li et al., 2020). The cAMP waves are initiated at the slug tip and propagate towards the back of the slug (Siegert and Weijer, 1993). Because of the primary tip dominance, additional tip formation is repressed, a phenomenon called tip inhibition and adenosine plays a crucial role in this process by inhibiting new tip formation (Schaap and Wang, 1986).

Caffeine is a purine alkaloid that is lipid-soluble, permitting it to easily cross biological membranes. In humans, caffeine acts as a non-competitive antagonist of adenosine receptors and inhibits phosphodiesterases and several proteins kinases of the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family, including PI3K and mechanistic target of rapamycin (mTOR)(Islam et al., 2019). Caffeine inhibits the oscillatory cAMP wave and removes tip inhibition by reducing the amplitude and oscillation frequency of cAMP signals. Caffeine, the antagonist of adenosine favors tip activation inducing multiple tip formation (Mac Williams, 1991).

RapC plays an important role in the tip formation from the aggregates at the late mound stage of development. Lacking of RapC resulted in aberrations in multicellular development after the normal aggregation stage, producing multi-tipped mounds and multi-branched developmental structures. The phenotypes such as increased cell spreading, multinucleation, and increased cell adhesion have been reported for several Rap1- related mutants. also, no studies have indicated in our knowledge that Rap signaling proteins are involved in multi-tip formation during development. Ras Overexpressing cells formed similar multiple tips and branched structures but were unable to progress to the following developmental process, whereas *rapC* null cells ultimately formed fruiting bodies after tip formation although progression was slightly delayed (Park et al., 2018b). I investigated the mechanism for multiple tip formation of cells lack RapC and examined the possibility that cAMP signaling pathways or membrane potential changes are involved in the regulation of morphogenesis during development.

II. MATERIALS AND METHODS

II-1. Development assay

Log-phase 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. Developmental phenotypes were examined at various concentrations of cAMP and potassium under a phase-contrast microscope.

II-2. Cell sorting assay

For examination of cell movement and differentiation in a multicellular organism, 3% of red fluorescent protein (RFP)-labeled wild-type cells and 3 % of GFP-labeled *rapC* null cells were mixed with 94 % of unlabeled wild-type cells and developed on Na/K phosphate agar plates for 6 h. Fluorescence images of developmental structures were captured by the NIS-Elements software and a fluorescence microscope.

III. RESULTS

III-1. Caffeine induces multiple tip formation.

Dictyostelium cells live in a single-cell state under nutrient-rich conditions. Depletion of nutrients from the medium triggers the cells to aggregate, which is mediated by chemotaxis of the cells to cAMP, and the cells finally form multicellular fruiting bodies over a period of 24 h. The cells within aggregates at the initial stage of development differentiate and sort into several types of cells, form slugs, culminate, and finally form fruiting bodies consisting of dead stalks with a mass of spores on the top.

Caffeine is an inhibitor for cAMP signaling pathways. To determine if the cAMP signaling pathway is involved in the formation of multiple times from an aggregate, I examined development in the presence of caffeine in the developmental media. *rapC* null cells formed aggregates at 12 h after starvation, multiple mounds at 16 h, and then multiple tips within 24 h. In the presence of 2 mM caffeine in the plates, wild-type cells showed developmental phenotypes similar to *rapC* null cells, forming multi-tipped mounds and multi-branched developmental structures (Fig 1). These data indicate that caffeine induces multiple tip formation, suggesting that cAMP signaling pathways are involved in the tip-forming stage during development since caffeine is known as an inhibitor for cAMP signaling pathways.

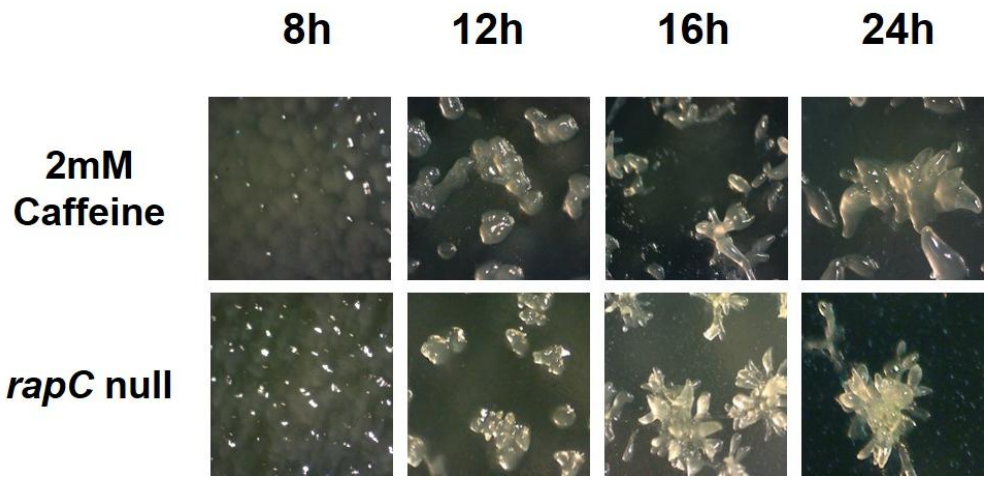

Fig 1. Development of the presence of caffeine.

Exponentially growing cells were washed and developed on non-nutrient agar plates, and another group of the cells were developed on the plates containing 2Mm caffeine. Photographs were taken at the indicated times after plating. Representative developmental images of the cells at 8 h (aggregation stage), at 12 h and 16 h (tip and slug formation stage), and at 24 h (fruiting body formation stage) are shown.

III-2. Distribution of *rapC* nell cells during development.

I examined the ability of *rapC* null cells to be polarized and migrated into the center of the aggregates during development. Development of chimeric cells, containing 94 % unlabeled wild-type cells, 3 % RFP expressing wild-type cells and 3% GFP expressing cells, were induced by removing the nutrients, and then the location of the labeled cells was examined in the aggregation stage of development (Fig 2). There was no difference at the aggregation stage (6 h) of development between wild-type cells and *rapC* null cells. However, GFP-labelled *rapC* null cells showed some accumulation at the tip of developmental structures and the spore (at 24 h).

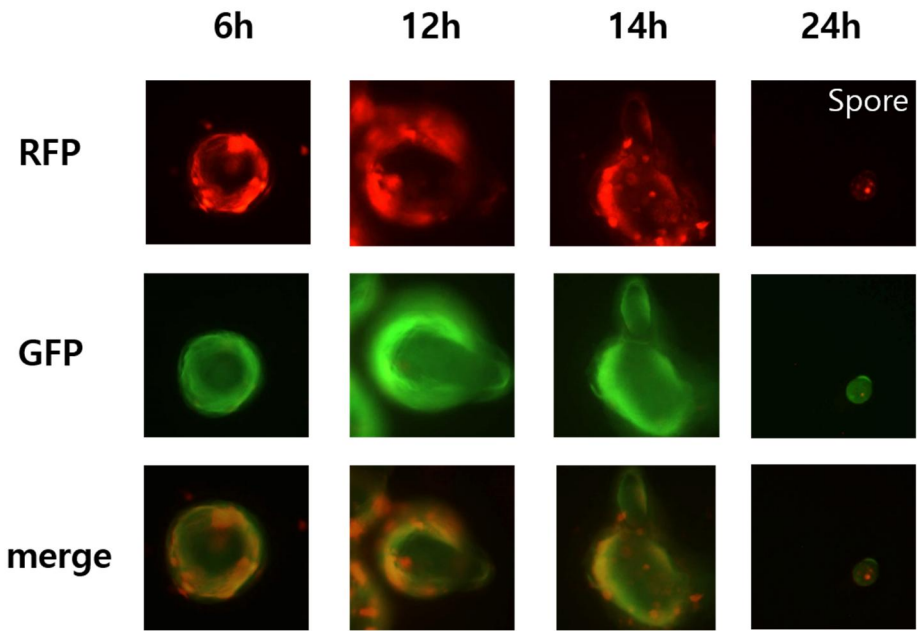

Fig 2. Distribution of *rapC* null cells during development

Distribution of the cells at the mound, slug, and fruiting body forming stages of development. 3% RFP-labeled wild-type cells, 3 % GFP-*rapC* null cells and 94 % unlabeled wild-type cells were mixed and developed. The images were taken at the indicated times.

III-3. Development of the cells in the presence of cAMP.

The previous experiments showed that caffeine treatment resulted in abnormal development, suggesting involvement of the cAMP signaling pathway. cAMP plays a role in development through regulating protein kinase A (PKA) and PKB. Thus, I investigated whether cAMP has an effect on multiple tip formation in *rapC* null cells. *rapC* null cells were developed in the presence of 1 μM cAMP and compared with *rapC* null cells. *rapC* null cells with or without cAMP in the media formed multiple tips, suggesting that cAMP in the media has no effect on development.

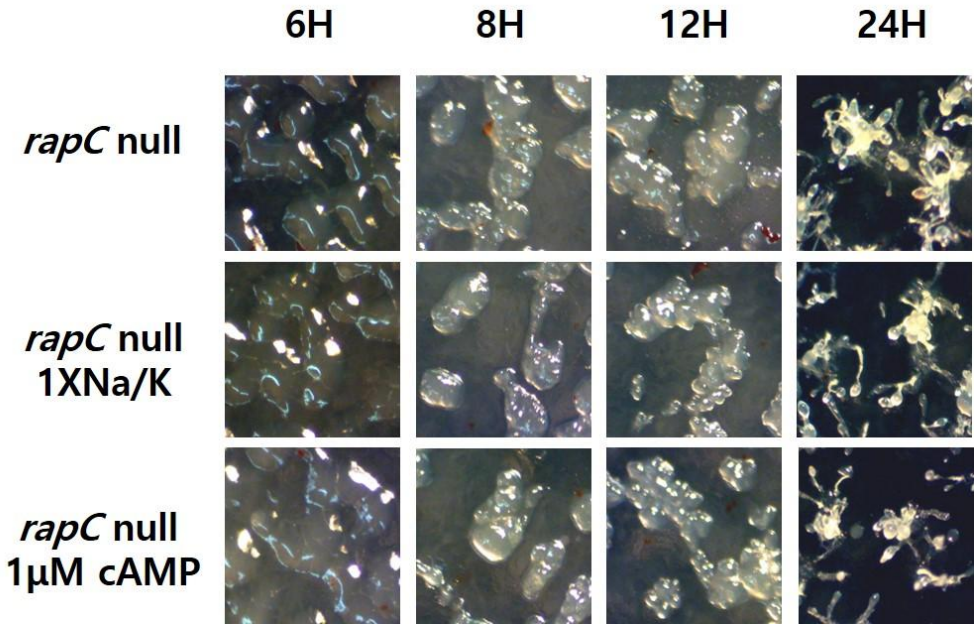

Fig 3. Development of *rapC* null cells in the presence of cAMP.

rapC null cells were developed on non-nutrient afar plates with or without 1 uM cAMP. 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 8 h (aggregation stage), at 12 h and 16 h (tip and slug formation stage), and at 24 h (fruiting body formation stage) are shown.

III-4. Development of the cells in the various conditions of membrane potential.

To determine if the membrane potential affects developmental processes, first I investigated whether there might be some differences in development in the various concentrations of potassium. I examined developmental processes of wild-type cells in the presence of 5, 50, and 100 mM potassium and compared with those in normal conditions (Fig 4). There was no clear difference when 5 mM potassium was added, but it seems that the developmental process was slightly delayed at 50 mM potassium. In the presence of 100 mM, multicellular development was not proceeded. When the cells were stimulated by electric fields, abnormal phenotypes were found in the tip-formation stage during development, multiple tips from an aggregate. Further examinations would be required to determine if the membrane potential is involved in development.

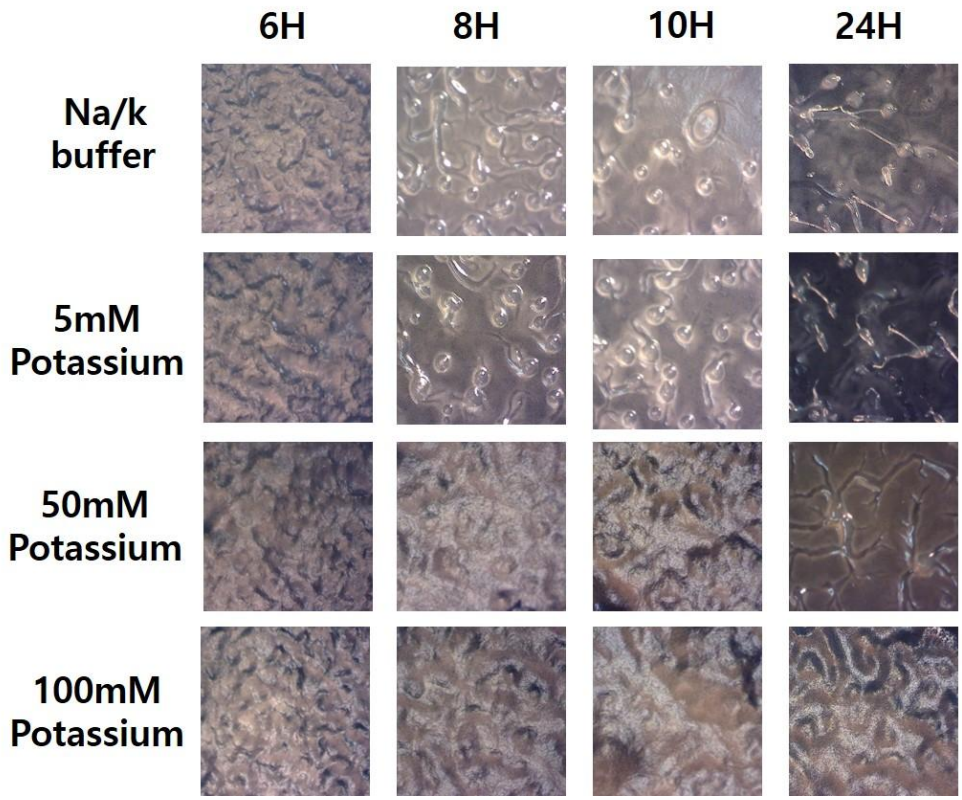

Fig 4. Development of the cells at the various concentrations of potassium.

The exponentially growing cells were washed and plated on the non-nutrient agar plates containing 5, 50, and 100 mM potassium. Developmental images of the cells at 8 h (Wild-type aggregation stage), at 12 h and 16 h (tip and slug formation stage), 24 h (wild-type fruiting body stage) are shown.

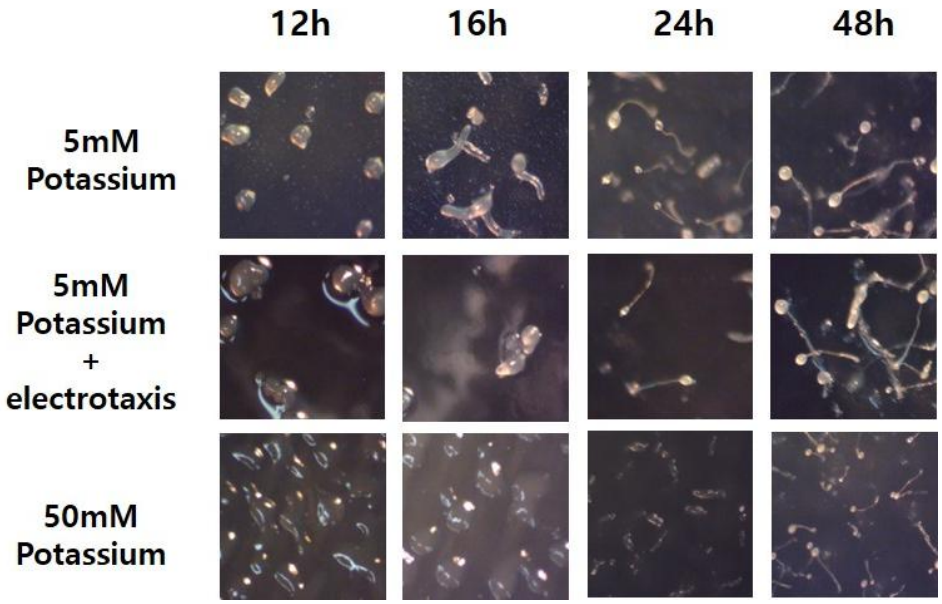

Fig 5. Development of the cells in the presence of electric fields.

The exponentially growing cells were washed and plated on the agar plate. 5 V/cm of electric filed was given on the plates in the presence of 5 mM potassium. 5 mM and 50 mM potassium were used as controls to compare the developmental phenotypes.

IV. DISCUSSION

RapC has a unique function in development compared to other Rap proteins. *rapC* null cells form multiple tips in development. These similar phenotypes were also found when the cells were the cells were treated with caffeine. These data suggest that *rapC* null cells might have a defect in cAMP signaling pathway since similarity in the phenotypes of development between cells lacking RapC and caffeine-treated cells. Further studies should be followed to determine if the membrane potential is involved in development processes, especially at the tip-formation stage. Potassium treatment affects on the membrane potential. I was unable to observe the development phenotypes, forming multiple tips, in the presence of high concentrations of potassium. However, there seems a few multiple tips formed when the cells were applied by electric fields in the presence of 5 mM potassium.

It has been reported that caffeine not only inhibits PKBR1 and PKA activity but also has a positive effect on RapA through the PKA-mediated negative feedback loop (Islam et al., 2019). RapA and RapC play antagonistic roles in cellular processes including cell morphology, adhesion, and cell migration (Jeon et al., 2007b; Park et al., 2018b). Formation of multiple tips of *rapC* null cells seems to be due to the PKA-mediated negative feedback loop. Whereas caffeine inhibits the basal and early cAMP-induced phosphorylation of ERK1 in cells, the cAMP-induced ERK2 phosphorylation response is increased. The activation levels of PKA and ERK1 in *rapC* null cells should be examined for further understanding the mechanism for multi-tip formation during development.

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먼저, 연구실 생활을 하면서 연구활동을 끊임없이 지지해 주시며 부족한 저를 이끌어 주시고 지도해 주신 전택중 교수님 정말 감사 드립니다. 항상 최선을 다하시며 저에게 여러 충고들이 저에게 가장 큰 힘이 되었던 것 같습니다. 또한 저의 논문을 심사해주시고 충고와 격려를 아끼지 않으신 조광원 교수님, 이준식 교수님 감사 드립니다. 복도에서 마주치는 짧은 시간에도 많은 조언과 따듯한 인사가 저에게 많은 힘이 되었습니다. 학부 때부터 많은 지도와 가르침 주신 윤성명 교수님, 조태오 교수님, 송상기 교수님, 이현화 교수님, 이현화 교수님, 원부연 교수님을 포함한 학과 교수님들께도 감사 인사 드립니다. 그리고 항상 지치고 어려움이 있을 때 커피를 주시며 항상 친누나처럼 다가와 조언 해 주신 김미은 박사님 정말 감사합니다.

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