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Characterization of Rap1GAPs and a Rap1 Downstream Effector FrmB in *Dictyostelium*

조선대학교 대학원

생명과학과

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Dictyostelium Rap1GAPs와
Rap1 하위 작동 단백질 FrmB의 기능 연구

2017년 2월 24일

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ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
cAR	Cyclic AMP receptor
FERM	Four-point-one, ezrin, radixin and moesin
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
MRL	MIG-10, RIAM and Lamellipodin
PH	Pleckstrin homology
PIP3	Phosphatidylinositol (3,4,5) trisphosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinases
RA	Ras association
RapGAP	Rap1-specific GTPase-activating protein
RBD	Ras-binding domain

ABSTRACT

Characterization of Rap1GAPs and a Rap1 Downstream Effector FrmB in *Dictyostelium*

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Cell movement involves the coordinated regulation of the cytoskeleton, F-actin-mediated protrusions at the front of the cell and myosin-mediated contraction of the posterior. The small GTPase Rap1 functions as a key regulator in the spatial and temporal control of cytoskeleton reorganization for cell migration. This study focuses on the mechanism for establishment of cell polarity by differential localizations of the cytoskeleton and spatial and temporal regulation of cytoskeleton reorganization via the Rap1 signaling pathway during chemotaxis. Cell migration requires a defined cell polarity, which is formed by diverse cytoskeletal components differentially localized to the poles of the cell. This study investigated Rap1-specific GTPase activating proteins and downstream effectors of Rap1.

RapGAP3 transiently and rapidly translocates to the cell cortex in response to chemoattractant stimulation and localizes to the leading edge of migrating cells. Part I of the present study examined the localization of truncated RapGAP3 proteins and found that the I/LWEQ domain in the central region of RapGAP3 was sufficient for posterior localization

in migrating cells, as opposed to the leading-edge localization of full-length RapGAP3. All truncated proteins accumulated at the leading edge of migrating cells exhibited clear translocation to the cell cortex in response to stimulation, whereas proteins localized to the posterior in migrating cells displayed no translocation to the cortex. The I/LWEQ domain appears to passively accumulate at the posterior region in migrating cells due to exclusion from the extended front region in response to chemoattractant stimulation rather than actively being localized to the back of cells. The results suggest that posterior localization of the I/LWEQ domain of RapGAP3 is likely related to F-actin. At the lateral and posterior regions of the cell there might be another type of F-actin with different properties compared to newly formed F-actin at the leading edge of migrating cells.

In addition to RapGAP3, in the previous studies on Rap1-specific GAP proteins, RapGAP1, RapGAPB, and RapGAP9 were identified and characterized. RapGAP1 was found to control the adhesion of cells at the leading edge of the cell during chemotaxis, while RapGAP3 was found to play an important role in the cell's developmental process. RapGAP9 was found to be associated with cell morphogenesis, adhesion, and cytokinesis. In Part II, I studied RapGAP6, one of the putative Rap GAP proteins expected to function as a Rap1 GAP protein. *rapGAP6* knockout cells and overexpressing cells were confirmed by PCR and Western blot analysis. RapGAP6, which is localized to the cytosolic vesicles, was found to be involved in cell-substratum adhesion and chemotaxis but not in development.

Phg1, Phg2, MyosinVII, and Talin were found to be Rap1 downstream effectors that all play similar roles in cellular adhesion. Part III of this study was focused on the Rap1 downstream effectors containing the RA domain. Phg2 and FERM-domain containing proteins are considered as Rap1 downstream effectors proteins since these proteins have a RA domain. Six proteins have been identified to have FERM domains in *Dictyostelium* : TalinA, TalinB, MyosinVII, FrmA, FrmB and FrmC. In the present work, to study the functions of FrmB, a

Rap1 downstream effector, FrmB knockout cells and overexpressing cells were created. FrmB was shown to be involved in cell adhesion, migration, and development. The results of this study would contribute to understand the Rap1 signaling pathway and several biological processes including cell migration and development.

국문초록

Dictyostelium Rap1GAPs와
Rap1 하위 작동 단백질 FrmB의 기능 연구

이미래

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세포이동은 세포 앞쪽 부분의 F-actin 매개성 돌출과 뒤쪽의 myosin 매개성 수축하는 세포골격 조절을 통해 일어난다. Small GTPase Rap1은 세포골격을 시간적, 공간적으로 재배열하여 세포이동 조절에 중요한 역할을 한다. 본 연구에서는 세포골격의 재배열에 의한 세포 극성화와 주화성 이동 시 Rap1 신호전달경로를 통한 세포골격의 시공간적 재배열에 대해 연구하였다. 세포이동은 다양한 세포골격 구성요소들이 세포의 서로 다른 극에 위치하게 되는 세포 극성화가 필요하다. 본 연구에서는 Rap1에 특이적인 비활성 단백질과 하위 작동 단백질에 대해 연구하였다.

RapGAP3는 주화성자극에 반응하여 빠르고 일시적으로 세포 피질로 위치이동하고 이동하는 세포의 앞쪽에 위치한다. Part I 에서는 RapGAP3 단백질

절편들의 위치를 확인하였다. RapGAP3의 전체 길이는 이동하는 세포의 앞쪽에 위치하지만, RapGAP3의 중앙에 위치한 I/LWEQ 도메인은 뒤쪽에 위치하는 것을 발견하였다. 이동하는 세포의 앞쪽에 축적되는 대부분의 단백질 절편들은 자극에 반응하여 세포 피질로 위치 이동한 반면, 뒤쪽에 위치하는 단백질들은 이러한 변화를 관찰할 수 없었다. 주화성자극에 대한 반응으로 I/LWEQ 도메인은 능동적으로 세포 뒤쪽에 위치하는 것과는 다르게, 뻗어나가는 앞쪽부분에 축적된 단백질이 사라지면서 이동하는 세포의 뒤쪽에서 수동적으로 축적하였다. RapGAP3의 I/LWEQ 도메인이 세포의 뒤쪽에 위치하는 것은 F-actin과 관련이 있다. 세포의 옆쪽과 뒤쪽 부분은 이동하는 세포의 앞쪽에 새롭게 형성되는 F-actin과는 다른 특성을 가진다.

RapGAP3 이외에도, 이전 연구에서 Rap1-특이적 GAP 단백질로 RapGAP1, RapGAPB 및 RapGAP9이 동정되었고 기능이 밝혀져 있다. RapGAP1은 주화성이동을 하는 세포의 앞쪽 가장자리에서 세포의 부착을 조절하는 것으로 알려져 있으며, RapGAP3는 세포의 발달 과정에서 중요한 역할을 한다. RapGAP9은 세포 형태 형성, 부착 및 세포질 분열과 연관되어 있다. Part II 에서는 Rap GAP 단백질들 중 하나인 RapGAP6 에 대해 연구하였으며, Rap1 GAP 단백질로서 기능을 할 것으로 예상된다. *rapGAP6* 결손 세포주와 과발현 세포주는 PCR 과 웨스턴 블롯을 통해 확인하였다. RapGAP6는 세포질 소포에 위치하고, 세포-바닥간 접착 및 주화성이동에는 관여하지만 발달에는 관여하지 않는 것을 발견하였다.

Phg1, Phg2, MyosinVII 및 Talin은 Rap1의 하위 작동 단백질로 모두 세포 부착에 대해서 유사한 역할을 하는 것으로 알려져 있다. 본 연구의 Part III 에서는 RA

도메인을 갖고 있는 Rap1 하위작동 단백질에 대해 연구하였다. Phg2 와 FERM 도메인을 포함하는 단백질들은 RA 도메인을 가지고 있어 Rap1 하위작동 단백질로 간주된다. *Dictyostelium* 에는 TalinA, TalinB, MyosinVII, FrmA, FrmB 및 FrmC를 포함한 6 개의 단백질이 FERM 도메인을 가지는 것으로 알려져 있다. 본 연구에서는 Rap1 하위작동 단백질인 FrmB를 연구 하기 위해, *frmB*의 결손 세포주와 과발현 세포주를 만들었다. FrmB는 세포 부착, 이동 및 발달에 관여하였다. 본 연구 결과는 Rap1 신호 전달 경로와 세포이동과 발달에 대한 생물학적 과정을 이해하는데 기여할 것으로 기대된다.

I. INTRODUCTION

1. Cell migration

Cell migration is involved in many biological and pathological processes, including embryonic development, wound healing, inflammatory responses, and tumor cell invasion and metastasis (Chung et al., 2001; Jin et al., 2009; Ridley et al., 2003). Thus, there is considerable interest in elucidating the fundamental mechanisms underlying cell migration. However, understanding cell migration is challenging because it requires the integration and temporal coordination of many different processes that occur in spatially distinct locations within the cell. The molecular machinery that controls cell migration is evolutionarily conserved between human leukocytes and simpler eukaryotic organisms (Chung et al., 2001; Stephens et al., 2008).

The social amoeba, *Dictyostelium discoideum*, has been used as a powerful 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 (Chisholm and Firtel, 2004; Kortholt and van Haastert, 2008; Williams et al., 2006). This process is very similar to macrophages or neutrophils chasing bacteria. Upon starvation, *Dictyostelium* undergoes a tightly regulated multicellular developmental process in which they secrete cyclic adenosine monophosphate (cAMP) and move toward cAMP via chemotaxis, leading to the eventual formation of fruiting bodies (Chisholm and Firtel, 2004).

The first step in chemotaxis is the binding of chemoattractants to cell surface G-protein coupled receptors (GPCRs). Upon ligand binding, GPCRs activate a series of signaling

pathways and molecules that induce migration toward the source of the chemoattractants (Kolsch et al., 2008; Kortholt and van Haastert, 2008). The basic migratory cycle includes extension of a protrusion in the direction of migration, formation of stable attachments near the leading edge of the protrusion, and release of adhesions and retraction of the posterior of a cell, leading to translocation of the cell body forward. The cell movement is mediated by a coordinated regulation of the cytoskeleton, F-actin-mediated protrusions at the front of the cell and myosin II-mediated contraction of the cell's posterior (Chung et al., 2001; Ridley et al., 2003).

2. Asymmetric distribution of the cytoskeleton during cell migration

Directional cell movement in response to chemoattractant stimulation requires a defined cell polarity in which cytoskeletal components are differentially localized at two poles of a cell. F-actin is polymerized at the front of the cell, leading to protrusion of the membrane surface and forward movement. This is followed by contraction of the cell's posterior, which results from myosin II-mediated contraction (Chung et al., 2001; Ridley et al., 2003) (Fig. 1). Regulation of myosin II assembly plays an important role in controlling the ability of cells to restrict the F-actin assembly site and pseudopod formation at the leading edge of moving cells (Chung et al., 2001; Jeon et al., 2007b; Kolsch et al., 2008; Kortholt and van Haastert, 2008).

(1) Assembly of F-actin at the leading edge

The highest concentration of F-actin is found at the leading edge of the cell, while a lower

concentration is found at the posterior. Rho family small GTPases are key regulators of F-acin assembly and adhesion and control the formation of lamellipodia and filopodia at the leading edge of moving cells. The most wellknown members of this family are the Rho, Rac, and Cdc42 proteins, which are present in all mammalian cells. Activated Rho proteins interact with their downstream target proteins, including protein kinases, lipid-modifying enzymes, and activators of the Arp2/3 complex, to drive cell motility. The major targets of Rac and Cdc42 that mediate actin polymerization in protrusions are the WASP/WAVE family proteins, which are Arp2/3 complex activators. Activated Rac proteins bind to WASP/WAVE proteins and stimulate the Arp2/3 complexes to induce dendritic actin polymerization (Firat-Karalar and Welch, 2011; Ridley et al., 2003; Rodal et al., 2005; Stephens et al., 2008). Recent studies have demonstrated that Rap proteins are interconnected with Rac signaling through interaction with the RacGEFs Vav2 and Tiam1 (Arthur et al., 2004) and the RhoGAPs Arap3 and RARhoGAP (Krugmann et al., 2006; Yamada et al., 2005). In mammalian cells, Rap1 controls cell spreading by mediating the functions of integrins and binding to and localizing Vav2 and Tiam1 to sites at which the cells are spreading (Arthur et al., 2004). Similar mechanisms have been found in the control of F-actin polymerization during chemotaxis in *Dictyostelium*, and recent studies have suggested that Rap1 is involved in the regulation of F-actin polymerization via direct binding to RacGEF1 (Mun and Jeon, 2012).

(2) Assembly of Myosin II at the posterior

Assembled myosin II is preferentially found in the rear body and along the lateral sides of moving cells in a decreasing posterior-to-anterior gradient. Assembled myosin II is required to maintain cortical tension along the lateral sides of cells, which prevents lateral pseudopod

formation (Chung et al., 2001; Ridley et al., 2003; Stephens et al., 2008). Myosin II is an important negative regulator of leading-edge function that restricts the site of pseudopod formation to the leading edge of moving cells (Lee et al., 2010; Meili et al., 2010). To maintain persistent motility, cells must release adhesive contacts from the substratum at their posterior and retract the rear body or uropod, which occurs via a process that depends on the motor activity of myosin II. In myosin II null *Dictyostelium* cells, retraction of the posterior of the cell during chemotaxis is defective and there is a loss of normal lateral cortical tension that leads to the formation of lateral pseudopodia and inefficient chemotaxis (Bosgraaf and van Haastert, 2006; De Lozanne and Spudich, 1987; Pasternak et al., 1989; Yumura et al., 2005). Myosin II is also localized in the uropod and involved in uropod retraction in fibroblasts and neutrophils (Worthylake and Burridge, 2003; Xu et al., 2003).

Dictyostelium myosin II, which is the most thoroughly studied conventional non-muscle myosin, has a molecular structure very similar to that of mammalian myosin II. The C-terminal coiled-coil regions of two myosin II monomers associate to form a bipolar dimer (Fig. 1). Assembly of these dimers into myosin filaments is regulated, in part, by phosphorylation of three threonine residues in the tail region by myosin heavy chain kinases (MHCKs), with phosphorylation resulting in filament disassembly and dephosphorylation in assembly (Bosgraaf and van Haastert, 2006; Kortholt and van Haastert, 2008) (Fig. 1B). In addition, regulation of myosin II assembly at the posterior of moving cells is mediated through PAKa and the cGMP pathway (Kortholt and van Haastert, 2008). Recent studies have demonstrated that Rap1 plays a key role in the spatiotemporal regulation of myosin assembly during cell migration in *Dictyostelium* (Jeon et al., 2007a; Jeon et al., 2007b), and that MHCK-A binds to F-actin and localizes to the leading edge of moving cells to help disassemble the myosin II fibers in this process (Steimle et al., 2001).

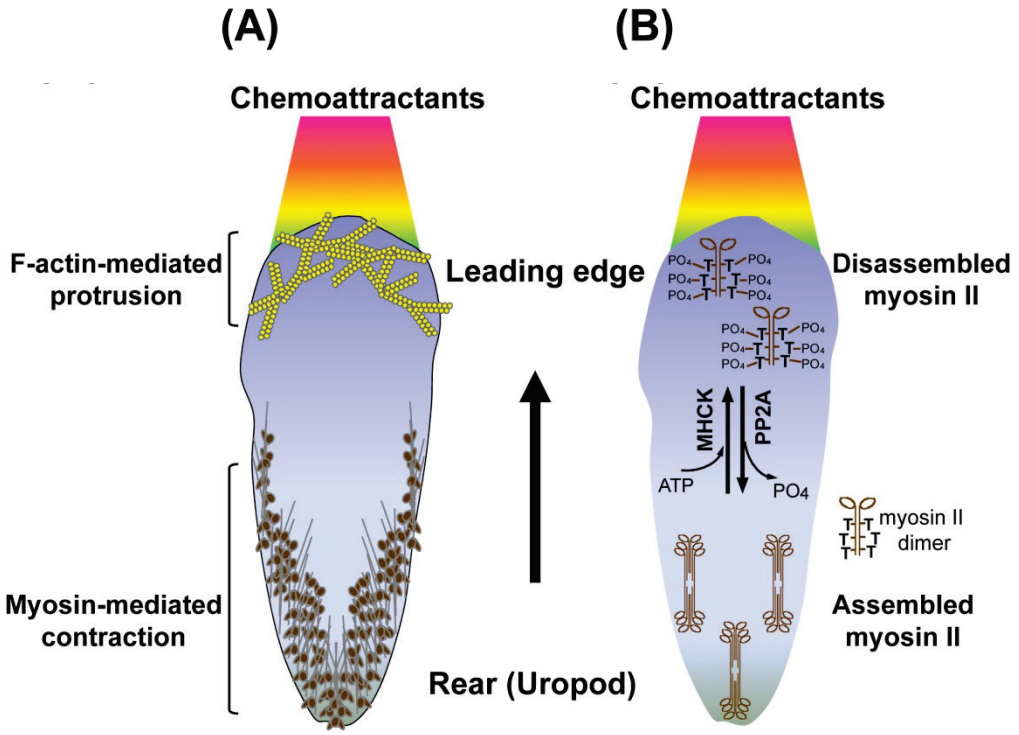


Fig. 1. Asymmetric distribution of the cytoskeleton.

(A) Differential localization of cytoskeletons during cell migration. F-actin-mediated protrusion at the leading edge and myosin mediated contraction at the posterior of a cell. The arrow indicates the direction of cell movement. (B) Regulation of *Dictyostelium* Myosin II assembly. Each myosin heavy chain contains a head domain and a tail domain, and two myosin heavy chains consist of a myosin II dimer (Bosgraaf and van Haastert, 2006). Phosphorylation on three threonines at the tail of the *Dictyostelium* myosin II by myosin heavy chain kinase (MHCK) facilitates disassembly of myosin II at the leading edge, while dephosphorylation by phosphatase PP2A leads to assembly at the posterior and lateral sides of a cell (Yumura et al., 2005).

3. Ras/Rap1 signaling pathways

Ras in *Dictyostelium*: Ras activation is one of the early responses 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 (Kortholt and van Haastert, 2008; Raaijmakers and Bos, 2009; Sasaki et al., 2004). The small GTPase Rap1 has recently been shown to play an important role in regulation of the cytoskeleton during cell migration.

Rap1 protein: Rap1 is the closest homologue of the small GTPase Ras and cycles between the inactive Guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP) bound forms. A variety of extracellular signals control this cycle through regulation of several unique guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Similar to Ras, Rap1 proteins function as molecular switches to control a wide variety of cellular functions, including integrin-meidated cell adhesion, cadherin-based cell-cell adhesions, cell polarity, cell proliferation, and cell survival (Kooistra et al., 2007; Kortholt and van Haastert, 2008; Raaijmakers and Bos, 2009).

A large number of proteins have been identified as effectors of Rap proteins in mammalian cells. The MIG-10, RIAM and Lamellipodin (MRL) family of adaptor proteins, which includes Riam and Lamellipodin, contains a Ras association (RA) domain

that interacts with Rap1-GTP and stimulates integrin-mediated cell adhesion and cell spreading (Krause et al., 2004; Lee et al., 2009). Other Rap1 effectors include the following: AF-6/Afadin, which is an adaptor protein that localizes to cell-cell junctions and binds p120 catenin in a Rap1-dependent manner to prevent internalization of E-cadherin (Hoshino et al., 2005); Krit1, which contains a four-point-one, ezrin, radixin and moesin (FERM) domain and controls endothelial cell-cell junctions (Glading et al., 2007); RAPL, which binds Rap1 after stimulation through the T-cell receptor or by chemokines and regulates LFA-1 (lymphocyte function-associated antigen 1) localization in a Rap1-GTP-dependent manner (Katagiri et al., 2006). PKD1 and IQGAP1 function as a scaffold protein by interacting with F-actin, which leads to the recruitment of Rap1 rather than activation by Rap1-GTP to induce downstream effectors of Rap1 (Medeiros et al., 2005). Interestingly, Rho family proteins play an important role in the process mediated by Rap1 and are directly linked to Rap1 signaling. For example, the RacGEFs Vav2 and Tiam1 interact with Rap1, resulting in localization of the RacGEFs to the sites of cell spreading (Arthur et al., 2004). Moreover, Arap3 is a RhoGAP containing five PH domains and an RA domain that interacts with Rap1 and affects PDGF-induced lamellipodia formation (Krugmann et al., 2006).

4. Regulation of cytoskeleton by Rap1 in *Dictyostelium*

In *Dictyostelium*, Rap1 has been linked to cytoskeletal regulation during cell migration, phagocytosis, and the response to osmotic stress (Jin et al., 2008; Kolsch et al., 2008; Kortholt and van Haastert, 2008). Recent studies using *Dictyostelium* have revealed that Rap1 plays important roles in the control of cell adhesion and spreading during cAMP-mediated chemotaxis. Cells expressing constitutively active Rap1 or lacking RapGAP1,

which has Rap1-specific GAP activity, are highly adhesive and unable to effectively regulate myosin II assembly and disassembly. As a result, these cells move slowly in chemotaxis and produce lateral pseudopodia more often than parental strains. In addition, they exhibit a flattened, spread shape, which is partially caused by an inability to spatially and temporally regulate myosin assembly and disassembly (Jeon et al., 2007b).

Rap1 is rapidly and transiently activated in response to chemoattractant stimulation with a peak at 5–10 sec. Activated Rap1 predominantly localizes at the leading edge of chemotaxing cells, whereas total Rap1 is primarily found on membrane vesicles and along the plasma membrane, suggesting that Rap1 plays some roles at the leading edge of chemotaxing cells (Cha et al., 2010; Jeon et al., 2007b) (Fig. 2). Rap1 has been shown to regulate cell adhesion and help establish cell polarity by locally modulating myosin II assembly and disassembly through Phg2, a Rap1-GTP-mediated Ser/Thr kinase that may control myosin heavy chain kinases. The Ser/Thr kinase Phg2 contains an N-terminal phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-binding domain, a Ser/Thr kinase domain, and a Rap1-GTP-binding RA domain that interacts preferentially with Rap1-GTP over Ras-GTP (Gebbie et al., 2004). Upon chemoattractant stimulation, Phg2 rapidly and transiently translocates to the cell cortex and localizes to the leading edge of polarized chemotaxing cells, which is similar to the temporal and spatial localization of Rap1-GTP (Fig. 2). An *in vitro* assay demonstrated that the kinase Phg2 is required for myosin II phosphorylation, which disassembles myosin II and facilitates F-actin-mediated leading edge protrusion. Based on these results, a model in which the recruitment and activation of Phg2 at the leading edge by Rap1-GTP are required for myosin II phosphorylation and disassembly at the newly formed pseudopod has been proposed. Rap1/Phg2 plays a role in controlling leading edge myosin II disassembly while passively allowing myosin II assembly along the

lateral sides and posterior of the cell (Fig. 1 and 2), providing cortical tension along the lateral sides of the cells and inhibiting F-actin-mediated protrusions. On the other hand, disassembly of myosin II at the leading edge by Rap1/Phg2 allows the cells to lead to F-actin-mediated protrusions at the leading edge (Jeon et al., 2007b; Kortholt and van Haastert, 2008; Lee et al., 2010; Meili et al., 2010). Regulation of myosin II assembly by Rap1 plays an important role in controlling the ability of cells to restrict the F-actin assembly site and pseudopod formation at the leading edge of moving cells.

Rap1 also appears to be directly involved in regulation of F-actin polymerization through the Rac signaling pathway. Our recent study showed that Rap1 interacts with RacGEF1 in vitro and stimulates F-actin polymerization at the sites at which Rap1 is activated upon chemoattractant stimulation (Mun and Jeon, 2012). Rac family proteins are crucial regulators in actin cytoskeletal reorganization. Cells expressing constitutively active Rap1 or *rapGAP1* null cells exhibit higher levels of F-actin than parental strains (Jeon et al., 2007b; Jeon et al., 2007a). An *in vitro* binding assay using truncated RacGEF1 proteins revealed that Rap1 interacts with the DH domain of RacGEF1 (Mun and Jeon, 2012), suggesting that probably Rap1 mediates F-actin polymerization by binding RacGEF1 and possibly activating RacB.

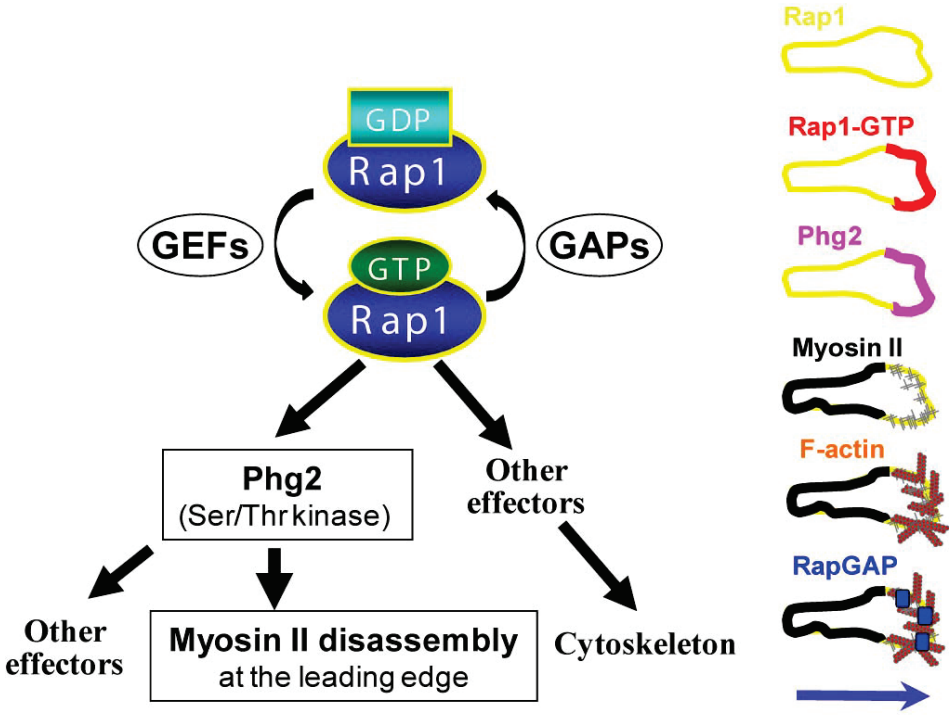


Fig. 2. Myosin II disassembly at the leading edge by Rap1/Phg2 pathway and localization of Rap1 signaling components during chemotaxis.

The activation of Rap1 is regulated by GEFs and GAPs. Activated Rap1 stimulates Ser/Thr kinase Phg2, leading to phosphorylation of myosin II and disassembly at the leading edge. Other downstream effectors of Rap1 might be involved in regulation of the cytoskeleton. The localizations of Rap1, Rap1-GTP, Phg2, myosin II, F-actin, and RapGAP are shown in yellow, red, pink, black, brown, and blue, respectively. The arrow below the drawing of the cell indicates the direction of cell movement.

5. Regulation of Rap1 activity during chemotaxis in *Dictyostelium*

Rapid and transient activation of Rap1 in response to chemoattractant stimulation plays an important role in control of cell adhesion and cytoskeleton reorganization. Chemoattractant-mediated Rap1 activation in *Dictyostelium* requires the G-protein coupled receptors, cAR1/cAR3 (cyclic AMP receptor), and G-proteins. Rapid Rap1 activation upon cAMP chemoattractant stimulation was absent in *Dictyostelium* cells lacking chemoattractant cAMP receptors cAR1/cAR3 or a subunit of the heterotrimeric G-protein complex, Gα2. Cells that are unable to form cGMP have no effect on Rap1 activation, suggesting that Rap1 activation upon chemoattractant stimulation is independent of cGMP signaling (Cha et al., 2010; Jeon et al., 2007a).

Rap1 GEFs: GbpD has been identified as a Rap1-specific GEF protein. Cells overexpressing GbpD are flat and exhibit strongly increased cell-substratum attachment and severely impaired chemotaxis. The phenotypes of cells expressing GbpD are similar to those of cells expressing Rap1. GbpD has been shown to activate Rap1 both *in vivo* and *in vitro* and to be involved in the formation of cell polarity. However, the mechanism by which GbpD is regulated remains unclear. Although GbpD contains two cyclic nucleotide binding domains, no binding of cAMP or cGMP to GbpD has been detected to date. Additionally, strong phenotypes of GbpD overexpressing cells are independent of the presence of cAMP/cGMP, indicating that the activity of GbpD is not strictly regulated by cyclic nucleotides (Bosgraaf et al., 2005; Goldberg et al., 2002; Kortholt et al., 2006). GbpC is a homologue of GbpD and the only known cGMP-binding protein. GbpC seems to be dispensable to Rap1 activation by cAMP chemoattractant stimulation since normal Rap1 activation kinetics were observed in GbpC deficient cells upon stimulation (Cha et al., 2010). The sequencing of *Dictyostelium* genome was recently completed, and 25 open reading

frames containing a putative RasGEF domain in the *Dictyostelium* genome were identified (Wilkins et al., 2005). However, the RasGEFs that have Rap1-specific GEF activity have not yet been identified.

Rap1 GAPs: The *Dictyostelium* genome contains nine ORFs that possess the Rap1 GAP domain (Jeon et al., 2007a). RapGAP1 is the first identified GAP protein specific to Rap1, and is required for regulation of cell adhesion by controlling Rap1 activity at the leading edge of chemotaxing cells. For a cell to keep moving, repeated cycles and coordinated regulation of cell adhesion and detachment are required. Rap1 is activated at the leading edge of moving cells and contributes to adhesion of the cell's front. Activated Rap1 is subsequently deactivated and the attached region of the cell is detached to enable the cell to move forward. RapGAP1 plays a key role in regulating Rap1-mediated adhesion at the leading edge of a cell during chemotaxis in *Dictyostelium*. Spatial and temporal regulation of Rap1 activity by RapGAP1 was examined using the Rap1-GTP reporter RalGDS-YFP and RFP-RapGAP1 (Jeon et al., 2007a) (Fig. 3). RapGAP1 transiently translocates to the cell cortex with a peak at ~10 sec upon chemoattractant stimulation, which is 2–4 sec slower than the translocation of RalGDS-YFP (Fig. 3A). The slightly delayed RapGAP1 cortical localization relative to that of Rap1 activation suggests that the kinetics of RapGAP1 localization may provide a timing mechanism that limits Rap1 activity. In chemotaxing cells, RapGAP1 preferentially localizes to the leading edge, which is similar to Rap1-GTP and consistent with its involvement in regulation of adhesion at the anterior of moving cells. Further examination has revealed that the localization of RalGDSYFP differs slightly from that of RapGAP1 (Fig. 3B). Specifically, RalGDS is always found at the leading edge plasma membrane, whereas RapGAP1 predominantly localizes to the region overlapping with and slightly posterior to this at sites of F-actin accumulation, providing a spatial

mechanism for limiting Rap1 activity by RapGAP1. The localization of RapGAP1 in chemotaxing cells is mediated by F-actin and actin-bundling proteins cortexillins (Jeon *et al*., 2007a, 2007b). Cortexillins play an inhibitory role in producing pseudopodia along the lateral sides of the cell. The localization of Cortexillin I at the lateral sides of moving cells is related to inhibited production of lateral pseudopodia, and cortexillins are linked to the translocation of Arp2/3 complex to the cell cortex upon chemoattractant stimulation (Cha and Jeon, 2011; Lee et al., 2010). Defects in spatial and temporal regulation of Rap1 activity and cell attachment at the leading edge in *rapGAP1* null cells or cells expressing RapGAP1 lead to defective chemotaxis. Cells lacking RapGAP1 have extended chemoattractant mediated Rap1 activation kinetics and decreased myosin II assembly, whereas those overexpressing RapGAP1 show reciprocal phenotypes. GFP-RapGAP1 overexpressing cells are unable to temporally and spatially regulate substratum attachments near the anterior of the cell immediately after pseudopod extension. In cells expressing GFP-RapGAP1, an extended anterior remains off of the substratum for a longer time, during which it randomly shifts direction relative to the chemoattractant gradient (Jeon *et al*., 2007a). RapGAPB and RapGAP3 have also been identified as Rap1 GTPase activating proteins in *Dictyostelium* and shown to be involved in the multicellular developmental process of *Dictyostelium*. RapGAPB is required for the correct sorting behavior of different cell types during development, but not cell motility or chemotaxis. Defects in RapGAPB affect prestalk and prespore cell adhesion, leading to abnormal morphogenesis of the multicellular organisms and misregulation of cell-type patterning during development in *Dictyostelium* (Parkinson *et al*., 2009). RapGAP3 mediates the deactivation of Rap1 during the late mound stage of development and plays an important role in regulation of cell sorting during apical tip formation by controlling cell-cell adhesion and cell migration. Direct measurement of cell

motility within the multicellular organism mound shows that *rapGAP3* null cells have a reduced motility toward the apex, resulting in severely altered morphogenesis during development (Jeon *et al*., 2009).

Rap1 plays important roles in the dynamic control of cell adhesion by regulating the cytoskeleton during cell migration. Identification and characterization of Rap1-specific GEFs and GAPs would provide further insights into the molecular mechanisms through which Rap1 controls cell adhesion during chemotaxis. To understand the spatial mechanism by which directs localization of RapGAP3 during migration, I examined the subcellular localization of truncated RapGAP3 proteins. Here, I present an analysis of the RapGAP3 fragments required for polarized localization of the protein in migrating cells. To understand functions of Rap1, in Part II examines RapGAP6 (DDB0233725), one of the putative Rap1 GAPs, and found that RapGAP6 is involved in morphogenesis, cell adhesion, and migration in *Dictyostelium*. To further understand functions of other Rap1 downstream effectors, I investigated that FrmB (DDB0233516) in Part III and found that FrmB is involved in cell adhesion and development in *Dictyostelium*.

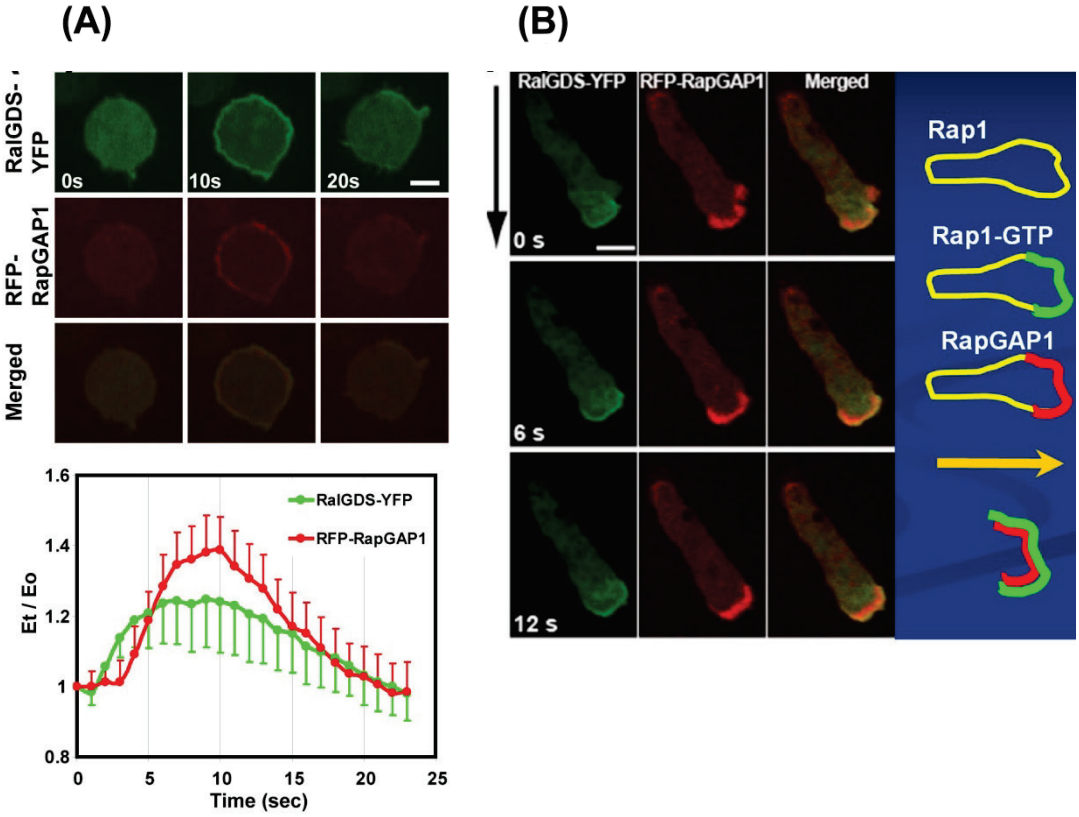


Fig. 3. Localization of Rap1-GTP and RapGAP1.

(A) Translocation of the two proteins to the cell cortex upon chemoattractant stimulation. Activated Rap1 and RapGAP1 were labeled by RalGDS-YFP and RFP, respectively. The intensity of the fluorescence of the protein at the cell cortex was quantified and shown in the lower panel. (B) Spatial localization of the activated Rap1-GTP and RapGAP1 during chemotaxis. Localizations of the activated Rap1-GTP and RapGAP1 are shown as green and red lines, respectively, on the right [Adapted from Jeon *et al*. (2007a)].

II. MATERIALS AND METHODS

(1) Cell culture

Dictyostelium wild-type KAx-3 cells were cultured axenically in HL5 medium or in association with *Klebsiella aerogenes* at 22°C. The knock-out strains and transformants were maintained in 10 μg/ml blasticidin or 20 μg/ml and 50 μg/ml of G418.

(2) Strains and plasmid construction

The *myosin II*, *gCA/sGC*, and *gbp a/b* null strains were obtained from the DictyBase Stock Center. The expression plasmids for GFP-coronin and RFP-coronin were described previously (Cha and Jeon, 2011; Jeon et al., 2007b). For expression of GFP-RapGAP3, the full coding sequence of *rapGAP3* cDNA was synthesized by RT-PCR, cloned into the *Bgl*II-*Xho*I site of pBluescript KS(−), sequenced, and subcloned into the expression vector pEXP-4(+) containing a green fluorescent protein (GFP) fragment (Jeon et al., 2009). For expression of the truncated RapGAP3 proteins shown in Part I Figure 1A, the regions in RapGAP3 marked in the diagram were amplified by PCR and cloned into the *Bgl*II-*Xho*I site of a pEXP-4 vector containing a GFP fragment. The plasmids were transformed into KAx-3 cells, and the cells were maintained in 20 μg/ml of G418, 50 μg/ml of hygromycin, or both as required.

The *rapGAP6* knockout construct was made by inserting the Blasticidin resistance cassette (bsr casstte) into *BamH*I site created at nucleotide 557-564 of *rapGAP6* cDNA and used for a gene replacement in KAx-3 parental strains. Randomly selected clones were screened for a gene disruption by PCR, which was then confirmed by RT-PCR. For expression of GFP-RapGAP6, the full coding sequence of *rapGAP6* was generated by PCR,

cloned into the *Bgl*II-*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. For expression of truncated RapGAP6 proteins, the various GAP or lacking GAP domain sequences were amplified by PCR and cloned into the *Bgl*II-*Xho*I site of a pExp-4(+) vector.

The *frmB* knockout construct was made by inserting the Blasticidin resistance cassette (bsr casstte) into *BamH*I site created at nucleotide 1030 of *frmB* cDNA and used for a gene replacement in KAx-3 parental strains. The *frmC* knockout construct was made by inserting the Blasticidin resistance cassette (bsr casstte) into *BamH*I site created at nucleotide 1524 of *frmC* cDNA and used for a gene replacement in KAx-3 parental strains. Randomly selected clones were screened for a gene disruption by PCR. For expression of GFP-FrmB, the full coding sequence of *frmB* was generated by PCR, cloned into the *Eco*RI-*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment.

(3) Cell Adhesion assay

Log-phase growing cells on the plates were washed with 1× Na/K phosphate buffer, and then the amount of 2×10^6 cells in 100 μl were placed on the 6 well culture dish for overnight. The cells were photographed and counted for calculating the total cell number. To quantify attached cell number, plates were then shaken at 150 rpm for 1h, after that the medium was removed. The number of attached cells in plates were counted (attached cells). Cell adhesion was presented as a percentage of detached cells compared with total cells.

(4) Development assay

The Development was performed as described previously (Jeon et al., 2009). Exponentially growing cells were harvested and washed twice with 12 mM Na/K phosphate

buffer (pH 6.1) and plated on Na/K phosphate agar plates at a density of 3.5×10^7 cells/cm^2. The developmental morphology of the cells was examined by photographing the developing cells with a phase-contrast microscope.

(5) Chemotaxis and image acquisition

The subcellular localization of proteins in response to chemoattractant stimulation was examined as described previously (Cha and Jeon, 2011; Jeon et al., 2007b). Vegetative cells were washed twice with Na/K phosphate buffer, resuspended at a density of 5×10^6 cells/ml in Na/K phosphate buffer, and pulsed with 30 μM cAMP at 6-min intervals for 5 h. The pulsed cells were placed on glass-bottomed microwell plates. For imaging chemotaxing cells, a micropipette filled with 150 μM cAMP was positioned near the cells for stimulation. Images of chemotaxing cells were taken at time-lapse intervals of 6 s for 30 min using an inverted microscope (IX71; Olympus, Japan) with a camera (DS-Fi1; Nikon, Japan). Cell migration was analyzed using Dunn Chemotaxis Chamber (Hawksley). The images of chemotaxing cells were taken at time-lapse intervals of 6 s for 30 min or 1 h using an inverted microscope. The data were analyzed by using NIS-elements software (Nikon).

(6) Quantitative analysis of membrane or cortical localization of GFP fusion proteins

It was performed as described previously (Jeon et al., 2007b; Sasaki et al., 2004). Aggregation competent cells were allowed to adhere to the plate for 10 min. Cells were then uniformly stimulated with cAMP by quickly pipetting 250 μl of 150 μM cAMP into the plate containing cells. Fluorescence images were taken at time-lapse intervals of 1 sec for 1 min using an inverted microscope. The frames were captured using NIS-elements software

(Nikon) and analyzed using ImageJ software (National Institutes of Health, USA). The intensity of fluorescence at the cell cortex was measured, and the level of cortical GFP was calculated by dividing the intensity at each time point (Et) by the intensity before stimulation (Eo).

(7) RT-PCR

The total RNAs from wild-type cells and *rapGAP6* null cells were extracted by using the SV Total RNA Isolation System (Promega), and the cDNAs were synthesized by reverse transcription with MMLV reverse transcriptase (Promega) using random hexamers and 5 μg of total RNAs. 5 μl of the cDNAs were used in the following PCR with 35 cycles employing gene-specific primers. The universal 18S ribosomal RNA specific primers were used as an internal control (Jeon et al., 2007b; Schroeder et al., 2001).

Table 1. Primers used in the present work.

Gene name	Primer	Sequence (5' → 3')
18s rRNA	18s rRNA - F	GTAATTCCAGCTCCAATAGC
	18s rRNA - R	GAACGGCCATGCACCAC
RapGAP6	I (TJ17) - F	CCCAGATCTATGAAAAGGAGCCAAAGC
	II (IJ13) - F	GTAAATTCAGGTGGTGAAC
	III (TJ19) - R	CCCGGATCCAAAAGATTACTATTGC
	IV (TJ22) - R	TGGTAAATTCATTGATGG
	V (HM4) - F	GTAGTGGTAATACCAACCGAC
	VI (TJ53) - R	AAAATTTTTTTTATCTAGAGGATC
	VII (HM3) - F	CCCAGATCTATGACTAAAGATTATAAATTTGG
	VIII (TJ18) - R	CCCCTCGAGTTATTTTAAGGTTGTTGTTAC
FrmB	I (OTJ60) - F	CCCGAATTCATGGAATCATCATTTGAAGC
	II (OTJ62) - F	GTAATTATCCATGTGAAGAGG
	III (TJ53) - R	AAAATTTTTTTTATCTAGAGGATC
	IV (OTJ63) - R	CCCCTCGAGAAATTATTTCTTTTTTGAATC
	V (IJ15) - R	TTTAATACTCGTATTCCC

F - forward, R - reverse.

(8) Rap1 pull-down assay

The Rap1-GTP binding domain (RBD) of mammalian RalGDS was expressed in *Escherichia coli* as a GST fusion protein as described previously (Franke et al., 1997). The purified GST-RBD of RalGDS was used for the detection of activated Rap1. Log-phase vegetative cells on the plates were washed twice and resuspended at a density of 1×10^7 cells/ml in Na/K phosphate buffer. The cells were collected and lysed by mixing with 400 µl 1× lysis buffer (100 mM Tris, pH 7.5, 300 mM NaCl, 50 mM $MgCl_2$, 20% glycerol, 1% NP-40, 2 mM DTT, 2 mM vandate, protein inhibitor cocktail). The lysates were centrifuged for 20 min, and the supernatants were incubated with 10 µg GST-RBD on glutathione–Sepharose beads at 4°C for 2h. The beads were washed two times and subjected to SDS-PAGE and Western blot analysis with an anti-GFP pAb. For control of the input amount of total Rap1 proteins, 30 µl of the cells were taken right after lysis of the cells.

III. THE MAIN SUBJECT

Part I. The I/LWEQ domain in RapGAP3 is required for posterior localization in migrating cells

1. Introduction

Directional cell movement in response to chemoattractant stimulation requires a defined cell polarity in which cytoskeletal components are differentially localized to both poles of cells. At the front of cells, F-actin is polymerized and leads to protrusions of the membrane surface, whereas in a coordinated manner, myosin II assembly occurs at the posterior of cells and mediates contraction. How these asymmetries are spatially organized and maintained is one of the central questions in understanding cell migration (Lee and Jeon, 2012; Ridley et al., 2003; Sanchez-Madrid and Serrador, 2009).

Cell polarity is formed by a series of signaling molecules, including the Ras, PI3Ks, and PTEN which are differentially activated upon ligand binding to surface receptors. One of the early responses to chemoattractant stimulation is the activation of Ras proteins. Activated Ras proteins are enriched at the leading edge of chemotaxing cells, where they locally activate signaling molecules (Lee and Jeon, 2012; Ridley et al., 2003). Phosphatidylinositol 3-kinases (PI3Ks) rapidly accumulate at the leading edge of cells in response to a chemoattractant, whereas PTEN becomes restricted to the sides and the rear. The reciprocal localization and activation of PI3K and PTEN lead to accumulation of PIP3 at the leading edge, which helps guide the local polymerization of F-actin as well as pseudopod extension at the leading edge of cells possibly by recruiting PH domain-containing proteins such as

PhdA, CRAC, and PKB (Kortholt and van Haastert, 2008; Ridley et al., 2003). Assembled myosin II is preferentially found in the rear body and along the lateral sides in a decreasing posterior-to-anterior gradient where it is involved in uropod retraction. Assembly of myosin II at the back of cells is mainly regulated by several signaling molecules, including PAKa, the cGMP signaling pathway, and Rap1 signaling pathway (Kortholt and van Haastert, 2008; Lee and Jeon, 2012).

The small GTPase Rap1 is involved in the control of diverse cellular processes, including integrin-mediated cell adhesion, cadherin-based cell-cell adhesions, and cell polarity in mammalian cells as well as cell adhesion, phagocytosis, and cell migration in *Dictyostelium* (Kooistra et al., 2007; Kortholt et al., 2010; Raaijmakers and Bos, 2009). Rap1 is rapidly and transiently activated at the leading edge of cells during cell migration in response to chemoattractant stimulation. Leading-edge activation of Rap1 regulates cell adhesion and helps establish cell polarity by locally modulating myosin II assembly and disassembly through the Rap1/Phg2 signaling pathway (Cha et al., 2010; Jeon et al., 2007a; Kortholt and van Haastert, 2008; Lee and Jeon, 2012; Mun and Jeon, 2012). Recent reports have demonstrated that spatial and temporal regulation of Rap1 activity by Rap1 GAP proteins is required for proper cell migration. RapGAP1 was identified as a specific GAP protein for Rap1 and is involved in the regulation of Rap1 activity in the anterior of chemotaxing cells to control cell-substratum adhesion and myosin II assembly during chemotaxis (Jeon et al., 2007b). RapGAPB and RapGAP3 are required for the correct sorting behavior of different cell types during development (Jeon et al., 2009; Parkinson et al., 2009). RapGAP3 mediates deactivation of Rap1 at the late mound stage of development and plays an important role in regulating cell sorting during apical tip formation, when the anterior-posterior axis of the organism is formed, by controlling cell-cell adhesion and cell migration. RapGAP3

transiently and rapidly translocates to the cell cortex in response to chemoattractant stimulation, which is dependent on F-actin polymerization, and localizes to the leading edge of migrating cells (Jeon et al., 2009; Lee and Jeon, 2012).

To understand the spatial mechanism by which directs localization of RapGAP3 during migration, I examined the subcellular localization of truncated RapGAP3 proteins and found that the I/LWEQ domain in the central region of RapGAP3 is required for posterior localization of the protein during cell migration. Here, I present an analysis of the RapGAP3 fragments required for polarized localization of the protein in migrating cells.

2. Results

1) Subcellular localization of truncated RapGAP3 fragments in migrating cells

RapGAP3 has Rap1-specific activity and plays an important role in the process of development by regulating cell-cell adhesion in multicellular organisms. RapGAP3 is recruited to the cell cortex transiently and rapidly in response to chemoattractant stimulation and localizes to the leading edge of migrating cells (Jeon et al., 2009). To further understand the roles of RapGAP3 in the regulation of Rap1 activity during migration and development in *Dictyostelium*, I further investigated the roles of RapGAP3 protein domains in regulating the localization of RapGAP3 during migration.

RapGAP3 contains three PH domains at the N-terminal as well as a GAP domain at the C-terminal region (Fig. 1A). In addition, another domain similar to the I/LWEQ domain, known as the F-actin binding domain and originally characterized in the analysis of Talin proteins (Brett et al., 2006; McCann and Craig, 1997), was found in the central region of RapGAP3 when the amino acid sequence of the central region of RapGAP3 was compared with those of TalinA and TalinB using a sequence analysis tool (block maker).

To investigate the role of each domain in RapGAP3 function during the developmental process and movement, I prepared a series of truncated fragments fused with GFP and analyzed localization of the fragments during chemotaxis moving up a gradient of cAMP chemoattractants (Fig. 1B). Full-length Rap-GAP3 localized to the leading edge of migrating cells as previously reported (Jeon et al., 2009). Surprisingly, removing the GAP domain from RapGAP3 resulted in reverse localization of the proteins from the front to the back of cells (Fig. 1B). Cells expressing full-length RapGAP3 (#624) displayed high accumulation of the proteins at the leading edge along with a small amount at the rear and sides of cells. In contrast, cells expressing the fragments without the GAP domain (#628 and #627) exhibited

disappearance of the protein at the leading edge and accumulation with a decreasing gradient from the rear to the front of cells. GFP-fusion protein containing the GAP domain alone was found in the cytosol. These results suggest that RapGAP3 contains all of the properties required for localization to each pole in cells, whereas the GAP domain plays a role in localizing proteins to the anterior of cells during migration but is not sufficient for anterior localization.

To characterize the region required for posterior localization of the proteins, I further examined the localization of the fragments truncated at the N-terminus. The PH domain alone (#626) was enriched at the leading edge. Cells expressing the PH domain-truncated protein (#636) showed high enrichment at the leading edge as well as slight accumulation at the posterior and sides of cells, similar to that of cells expressing full-length RapGAP3. This result suggests that the PH domain functions in anterior localization of the protein but not required for localization to the front. It appears that there is an additional region in RapGAP3, possibly the GAP domain since GAP domain-deleted fragments were not found in the anterior region, functions in anterior localization during cell migration. Interestingly, the I/LWEQ domain without the PH or GAP domain (#137) was found at the posterior of migrating cells, similar to GAP domain-truncated proteins, indicating that the I/LWEQ domain alone is sufficient for posterior localization. Our results demonstrate that three regions, the PH, I/LWEQ, and GAP domains, contribute to the localization of RapGAP3 during migration. Further, the PH and GAP domains play roles in the anterior localization of RapGAP3, whereas the I/LWEQ domain is sufficient for posterior localization. The leading-edge localization of RapGAP3 suggests that the I/LWEQ domain in the full-length RapGAP3 might be masked by other domains and is unlikely to interact with the binding partners.

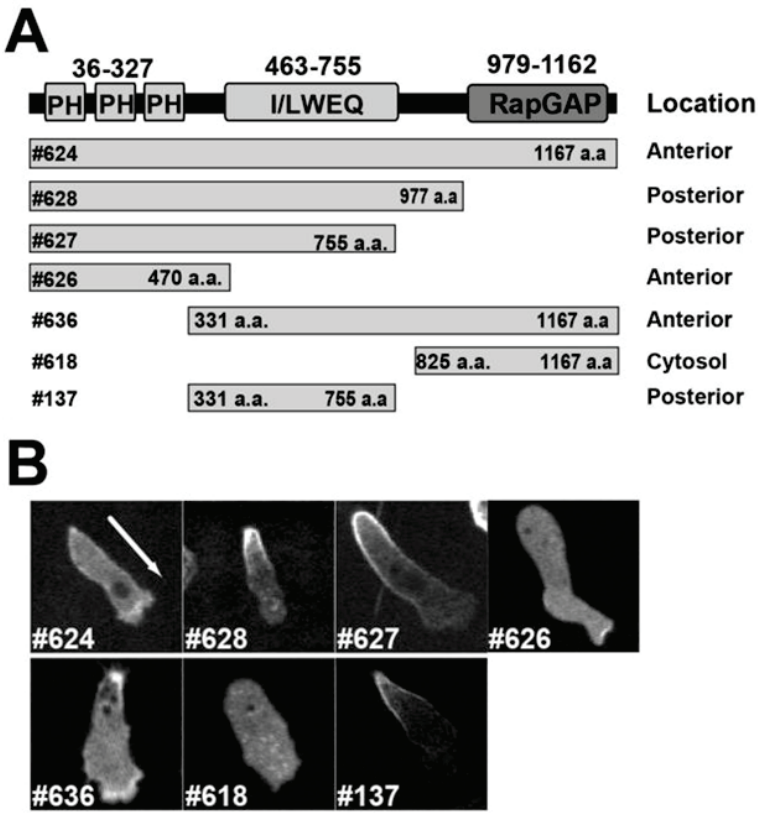


Fig. 1. Spatial localization of truncated RapGAP3 proteins in migrating cells.

(A) Schematic diagram of truncated RapGAP3 proteins. The locations of the proteins during chemotaxis are described at the end of each fragment. (B) Localization of the fragments in chemotaxing cells. The arrow indicates the direction of movement.

2) Translocation to the cell cortex in response to chemoattractant stimulation

RapGAP3 transiently and rapidly translocates to the cell cortex in response to chemoattractant stimulation with a peak of around 10 s (Jeon et al., 2009). I examined the translocation of truncated fragments of RapGAP3 to the cell cortex and analyzed translocation kinetics to further understand the factors affecting localization of RapGAP3. While full-length RapGAP3 (#624) showed transient translocation to the cell cortex with a peak at around 10 s after stimulation, the translocation of GAP domain-deleted RapGAP3 (#627 and #628 data not shown) to the cell cortex was not obvious (Fig. 2A and 2B). This result indicates that the GAP domain is required for translocation to the cell cortex in response to chemoattractant stimulation. The PH domain alone (#626), which was previously reported to transiently translocate to the cell cortex, exhibited translocation to the cell cortex upon chemoattractant stimulation, and PH domain-deleted fragments containing the GAP domain also showed clear translocation to the cortex. The I/LWEQ domain alone (#137) showed no clear translocation upon uniform stimulation, as GAP domain-deleted proteins. These results suggest that the PH and GAP domains are involved in the translocation of RapGAP3 to the cell cortex in response to chemoattractant stimulation, and the GAP domain has a main function in translocation since deletion of the GAP domain but not PH domain prevented translocation of the proteins.

More importantly, translocation of the proteins to the cortex upon uniform chemoattractant stimulation is likely to be correlated to the spatial localization of the proteins in migrating cells. All proteins accumulated at the leading edge of migrating cells (#624, #626, and #636) exhibited clear translocation to the cell cortex in response to uniform chemoattractant stimulation, whereas proteins localized to the posterior in migrating cells (#628, #627, #137) displayed no translocation to the cortex. Leading-edge localization

appears to be correlated to the transient accumulation of proteins by chemoattractant stimulation. These results suggest that leading-edge accumulation of the proteins (PH and GAP domains) might be actively induced in response to chemoattractant stimulation, whereas posterior localization of proteins (I/LWEQ domain) might be passively localized by binding to preexisting cytoskeletal components and excluded from the anterior by actively induced proteins.

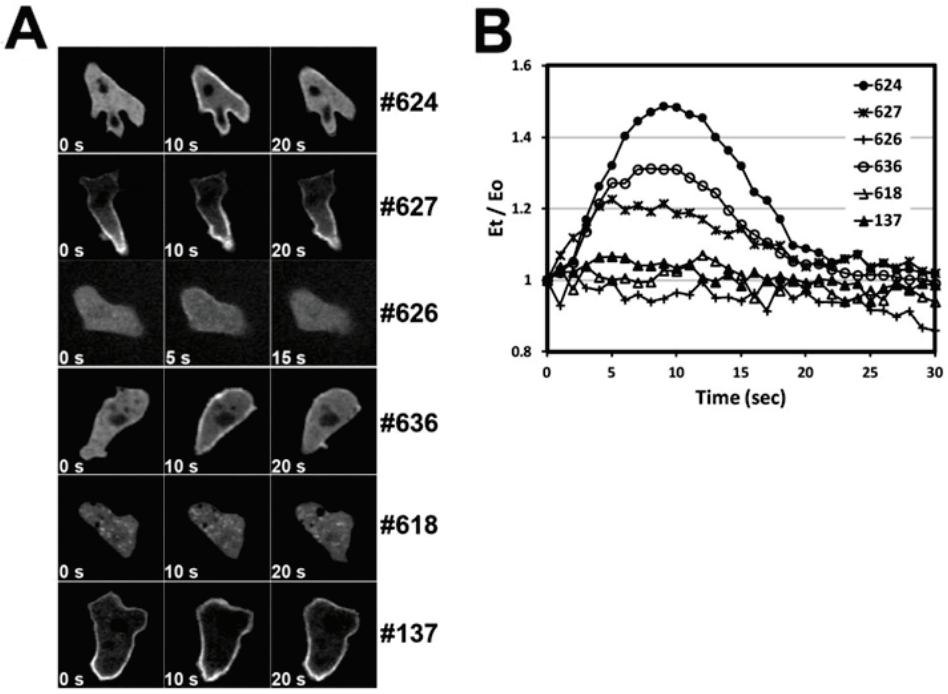


Fig. 2. Temporal translocalization of the truncated RapGAP3 proteins to the cortex.

(A) Translocation to the cell cortex in response to chemoattractant stimulation. Cells expressing truncated RapGAP3 proteins were uniformly stimulated with cAMP, after which the images were recorded. Representative images at 0, 10, and 20 s are presented. (B) Translocation kinetics of the fragments of RapGAP3 to the cell cortex from time-lapse recordings. Fluorescence intensity at the cell cortex was quantitated as described previously (Jeon et al., 2007a). Graphs are the means of several cells from videos from at least three separate experiments.

3) Localization of the I/LWEQ domain

The I/LWEQ domain was originally identified on the basis of sequence similarity in several F-actin binding proteins and has been shown to mediate cytoskeletal localization and F-actin binding (Brett et al., 2006; McCann and Craig, 1997; Senetar et al., 2004). Even though the I/LWEQ domain in RapGAP3 is expected to differentially accumulate at the front of migrating cells, in our previous localization study, the I/LWEQ domain of RapGAP3 was found at the rear and was shown to be sufficient region for posterior localization. To further understand the mechanism behind the asymmetric distribution of the protein during migration, I examined localization of the I/LWEQ domain of Rap-GAP3 in more detail (referred to Localization domain, LD).

In vegetatively growing cells, the LD was localized at the cell cortex. Interestingly, fluorescence of this protein decreased at protruding regions (Fig. 3A, indicated by arrows), where it is known that F-actin is polymerized and F-actin binding proteins usually accumulate (Kolsch et al., 2008; Ridley et al., 2003). This result is unexpectedly contrary to the expectation that the I/LWEQ domain might bind to F-actin and become localized to the front of migrating cells. It seems that the LD was excluded by the newly formed F-actin at protruding regions rather than binding to F-actin. However, controversial data in line with the notion that the I/LWEQ domain is excluded from F-actin at protruding regions shown in Fig. 3. It is known that cells form actin foci at their bottoms, which are exhibited by GFP-coronin, a marker protein for newly formed F-actin (Fig. 3B) (Cha and Jeon, 2011; Jeon et al., 2007b). GFP-LD was found at foci at the bottom of cells, similar to the actin foci shown by GFP-coronin. In addition, in the presence of Latrunculin A, an inhibitor of F-actin polymerization, most GFP-LD dissociated from the cell cortex, suggesting that the I/LWEQ domain localizes to the cell cortex in an F-actin dependent manner (Fig. 3C). The

controversial result that the I/LWEQ domain seems to be excluded from the protruding region by newly formed F-actin along with the occurrence of F-actin dependent localization to the cell cortex and actin foci at the bottom of cells raises the possibility of two more types of F-actin with different properties.

In moving cells, dynamic localization of GFP-LD was investigated by changing the location of the pipette filled with chemoattractants (Fig. 4A). The pipette filled with chemoattractants was placed at a position displaying a high level of GFP-LD near the cells (0 sec). Shortly after positioning of the pipette, the cells produced protrusions toward the pipette while, at the same time, the intensity of the fluorescence decreased at protruding regions (9 s and 24 s). It shows that the amount of GFP-LD at the cortex was rapidly diluted upon extension of the cell cortex toward the pipette, after which the proteins close to the pipette almost disappeared while the other side of cells showed reverse accumulation of GFP-LD. When the pipette was changed to another position (27 s), a high level of GFP-LD at the cell cortex close to the pipette (front of moving cells) disappeared as previously shown. It appears that GFP-LD passively accumulated at the posterior region in migrating cells due to exclusion from the extended front region in response to chemoattractant stimulation rather than actively being localized to the back of cells. It is known that F-actin accumulates at each pole of dividing cells, whereas myosin localizes to the cleavage furrow (Lee and Jeon, 2012; Ridley et al., 2003). The I/LWEQ domain was found at the cleavage furrow as well (Fig. 4B).

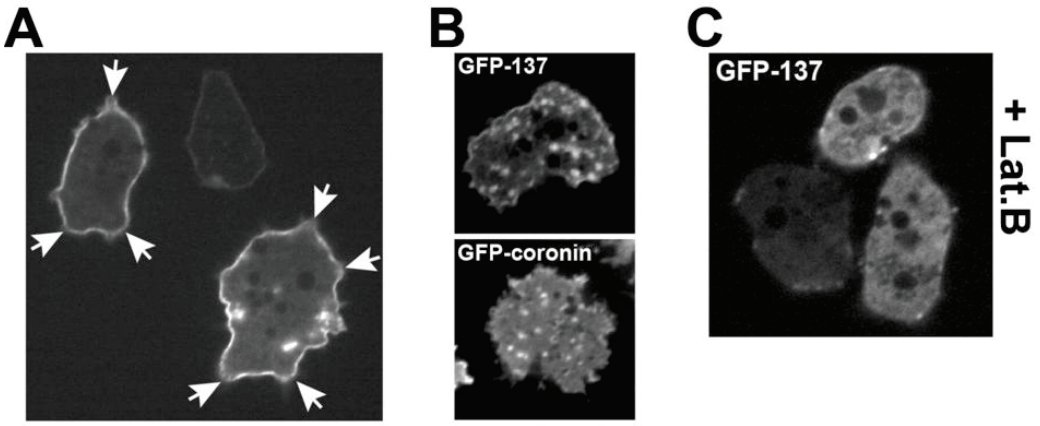


Fig. 3. Localization of the I/LWEQ domain in vegetative cells.

Cells expressing GFP-137 plasmid, which contains the I/LWEQ domain, were analyzed. (A) Localization of the I/LWEQ domain in vegetative cells. The arrowheads indicate the protruding regions. (B) Actin foci at the bottom of cells expressing the I/LWEQ domain (GFP-137) or GFP-coronin, which is a marker protein for newly formed F-actin. (C) Localization of the I/LWEQ domain in the presence of LatA, which is an inhibitor of F-actin assembly.

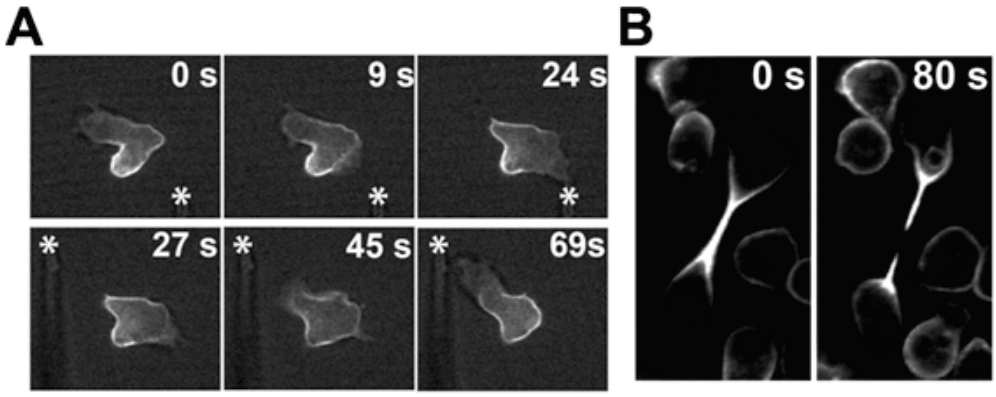


Fig. 4. Spatial localization of the I/LWEQ domain in migrating cells.

(A) Localization of the I/LWEQ domain (GFP-137) in chemotaxing cells upon changing the position of the micropipette filled with the chemoattractant cAMP. The asterisk indicates the location of the micropipette. (B) Localization of the I/LWEQ domain during cytokinesis.

4) Colocalization of the I/LWEQ domain with F-actin cytoskeleton

To gain insights into the relationship between the I/LWEQ domain and F-actin, we expressed both GFP-LD and RFP-coronin, which is a marker protein for newly formed F-actin, and then examined their dynamic localization in moving cells and translocation kinetics to the cell cortex in response to chemoattractant stimulation simultaneously. Cells expressing GFP-LD showed foci at the bottom of cells in the previous experiment. To determine if foci found in cells expressing GFP-LD are actin foci or colocalize with those exhibited in cells expressing RFP-coronin, I examined the bottom of cells expressing both GFPLD and RFP-coronin. As expected, RFP-coronin as well as GFP-LD were found at actin foci at the bottom of cells, and the locations of GFP-LD and RFP-coronin appear to exactly match in the merged images (Fig. 5A). This indicates that the foci shown by GFP-LD are localized to actin foci, and GFP-LD and RFP-coronin colocalize at least at the bottom of cells.

Next, I compared the translocation kinetics of both GFP-LD and RFP-coronin to the cortex (Fig. 5B). RFP-coronin exhibited transient translocation to the cell cortex, implying that F-actin newly and transiently formed at the cortex in response to chemoattractant stimulation, but no such translocation to the cortex was observed for GFP-LD. Compared to RFP-coronin, a high level of GFP-LD was found in unstimulated cells, and this high level was continually present at the cortex without alteration of fluorescence intensity upon chemoattractant stimulation. The merged images clearly show the differences in protein levels at the cell cortex. These results suggest that the localization of GFP-LD at the cell cortex is not related to newly formed F-actin.

To understand spatial localization of the two proteins, I examined the locations of the proteins in migrating cells (Fig. 6). In not-yet polarized cells, the two proteins GFP-LD and

RFP-coronin were found at partially overlapped regions at the cortex. A small difference in their localization was found at the protruding regions of non-moving cells. RFP-coronin accumulated at protruding regions, indicating F-actin was newly assembled, whereas GFP-LD was present at a relatively low level compared with other cell regions. In moving cells, RFP-coronin differentially localized to the leading edge, in which F-actin is mainly polymerized. In contrast, the GFP-LD level was lower at the front of cells close to the pipette filled with chemoattractants, resulting in a decreasing localization gradient from the posterior to the front of cells. These results suggest that GFP-LD might be excluded from the leading edge of migrating cells by newly formed F-actin.

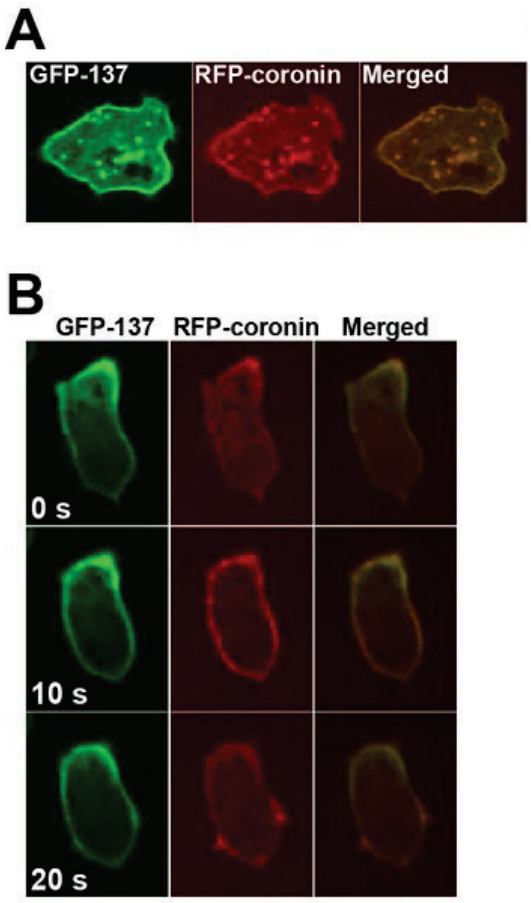


Fig. 5. Colocalization of the I/LWEQ domain with coronin.

Dual-view analyses of cells expressing both GFP-137 (the I/LWEQ domain) and RFP-coronin, which is a marker protein for newly formed F-actin. (A) Bottom sections of cells. (B) Translocation of both proteins to the cell cortex in response to chemoattractant stimulation was imaged.

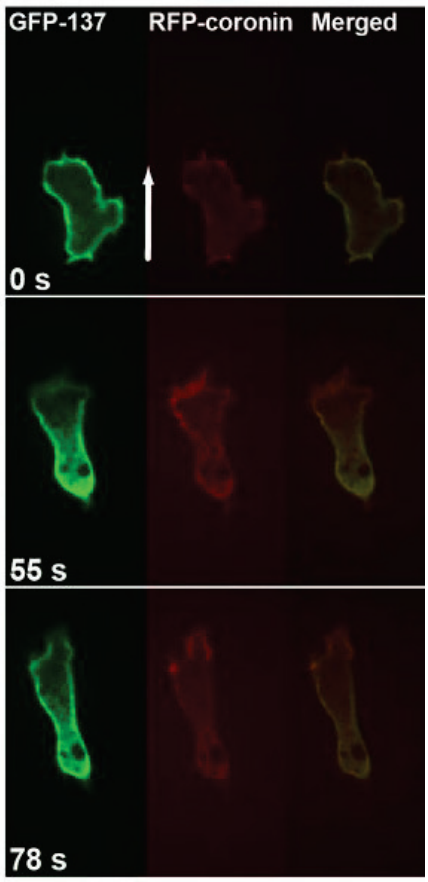


Fig. 6. Colocalization of the I/LWEQ domain and coronin in migrating cells.

Localization of both GFP-137 (the I/LWEQ domain) and RFP-coronin proteins during chemotaxis. The arrow indicates the direction of the cell.

5) Localization of the I/LWEQ domain in mutants

To determine which signaling pathways are involved in the posterior localization of LD in moving cells, I introduced GFP-LD into *myoII*, *gCA/sGC*, and *gbp a/b* null cells and examined localization of the protein in mutants (Fig. 7). As in wild-type cells, GFP-LD was localized to the posterior region in all mutant cells, suggesting that Myosin II and the cGMP pathway are not involved in localization of GFP-LD in the posterior of cells during migration. In the presence of LY, which is an inhibitor of PI3Ks, GFP-LD exhibited normal posterior localization as in control cells, suggesting that localization of GFP-LD is independent of the PI3K pathway.

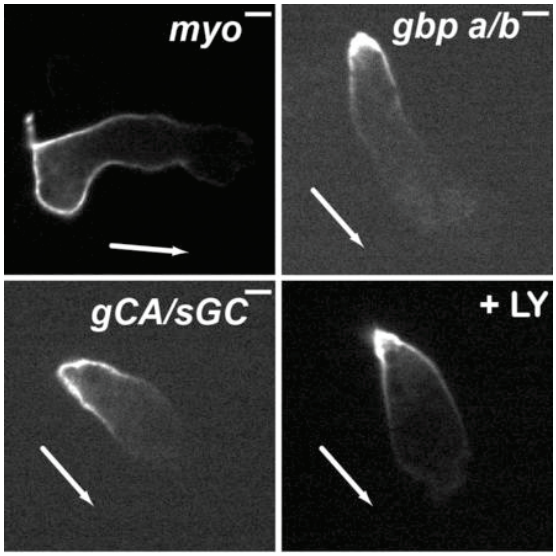


Fig. 7. Localization of the I/LWEQ domain in mutants.

The I/LWEQ domain (GFP-137) was introduced into myosin II null cells and two cGMP mutants, *gCA* (guanylyl cyclase A)/*sGC* (soluble guanylate cyclase) null cells and cGMP-specific phosphodiesterase null cells, after which localization of the domain in migrating cells was analyzed. In addition, localization of the I/LWEQ domain in wild-type cells pretreated with the inhibitor LY294002 was also analyzed.

3. Discussion

In this study, I investigated the localization of RapGAP3 fragments in migrating cells and found that the I/LWEQ domain in the central region of RapGAP3, which is known as an actin binding region and originally identified by comparison with Talin proteins (Brett et al., 2006; McCann and Craig, 1997; Senetar et al., 2004), localized to the posterior, even though full-length RapGAP3 was found at the anterior of cells during migration. The GAP domain was required but not sufficient for localization to the anterior of cells and transient translocation to the cell cortex in response to chemoattractant stimulation. Cell cortex localization of the I/LWEQ domain appeared to be dependent upon F-actin since the domain dissociated from the cortex after disruption of F-actin in the presence of LatA. On the basis of our results, posterior localization of the I/LWEQ domain is unlikely to be related to the cGMP pathway, myosin II, or PI3K pathway. *myosin II* null cells and cells with a disrupted cGMP pathway showed posterior localization of the I/LWEQ domain as in wild-type cells. Further, PI3Ks and the products of PI3K PIP3 had no effect on localization of the domain during migration.

For a cell to migrate, F-actin is assembled at the front of cells, producing an extension toward chemoattractants through a series of F-actin binding proteins such as coronin, Arp2/3 complex, and actinin, which are recruited to the leading edge of cells (Kortholt and van Haastert, 2008; Lee and Jeon, 2012; Ridley et al., 2003). In our study, coronin, used as a marker for newly formed F-actin, accumulated at the front of cells along with the bottom as foci. The I/LWEQ domain in RapGAP3 was also found at foci at the bottom of cells, which suggests that, along with its F-actin dependent cell cortex localization, the domain belongs to a family of F-actin binding proteins. However, the I/LWEQ domain was localized to the posterior but not anterior region, as with an F-actin binding protein.

To explain the result of posterior localization of the I/LWEQ domain as an F-actin binding protein in migrating cells, I propose that there might be two different types of F-actin with different binding affinities. One is newly formed F-actin filament at the front of cells while the other is pre-existing F-actin at the rear and lateral sides of cells. Our data suggest that the I/LWEQ domain may bind only to the pre-existing F-actin but not the newly formed F-actin at the front. Recent papers have reported a structural polymorphism in F-actin. F-actin is known to have a variable twist as well as a variable tilt of subunits (Galkin et al., 2010). It has been suggested that different actin-binding domains have different affinities for F-actin filaments in functionally distinct regions of the cytoskeleton, based on the studies about localization of the actin-binding domains of Filamin, which localizes to the rear of polarized cells, and actinin, which is enriched in new pseudopods and at the front of cells (Washington and Knecht, 2008). Myosin II motor domain localizes to the posterior of cells during migration (Jeon et al., 2007a; Kortholt and van Haastert, 2008; Lee and Jeon, 2012). It has been suggested that myosin II preferentially binds to stretched actin filaments in the rear cortex and cleavage furrows, and stretching of the actin filament itself increases its affinity for the myosin II motor domain (Kortholt and van Haastert, 2008; Uyeda et al., 2011). The I/LWEQ domain of Rap-GAP3 might have preferential binding affinity for stretched F-actin in the rear cortex and lateral sides of cells as opposed to the cross-linked, newly formed F-actin in the front of cells, helping establish cell polarity by locally modulating Rap1 activity. At the leading edge, actin is assembled as a dendritic network forming a lamellipodial shape (Galkin et al., 2010; Ridley et al., 2003). In contrast, at the posterior region, F-actin is oriented parallel to the long axis of migration, which is consistent with the specific localization of the actin-binding proteins Spectrin (Medina et al., 2002; Wang et al., 1999), Talins (Tsujioka et al., 2012; Weber et al., 2002), myosin II (Jeon et al., 2007a; Meili et al.,

2010; Uyeda et al., 2011), and the I/LWEQ domain of RapGAP3.

The polarity of cells is formed by diverse signaling molecules localized to the poles of cells in response to extracellular signals. Our results show that one type of protein, RapGAP3, has the ability to localize to either of the two poles. The I/LWEQ domain, which is known as an F-actin-binding region, in the central region of RapGAP3 was sufficient for posterior localization in migrating cells, whereas the GAP domain was required for anterior localization. The I/LWEQ domain appears to localize to the cell cortex in an F-actin dependent manner. However, it seems that the I/LWEQ domain was excluded by the newly formed F-actin at protruding regions rather than binding to F-actin. These controversial results raise a possibility that there are two different types of F-actin with different binding affinities. I suggest that the I/LWEQ domain of RapGAP3 might bind only to preexisting F-actin at the lateral and posterior regions of the cell but not to newly formed F-actin at the leading edge. Additional studies would be helpful to determine whether or not the I/LWEQ domain binds to newly formed F-actin or preexisting F-actin. This study provides new insights into the posterior localization of signaling molecules in response to chemoattractant stimulation.

Part II. RapGAP6 is involved in cell adhesion and migration

1. Introduction

The *Dictyostelium* genome contains nine ORFs that possess the Rap1 GAP domain (Jeon et al., 2007a). Rap1 is activated at the leading edge of moving cells and contributes to adhesion of the cell's front. Activated Rap1 is subsequently deactivated and the attached region of the cell is detached to enable the cell to move forward. RapGAP1 is the first identified GAP protein specific to Rap1, and is required for regulation of cell adhesion by controlling Rap1 activity at the leading edge of chemotaxing cells in *Dictyostelium* (Jeon et al., 2007a). In chemotaxing cells, RapGAP1 preferentially localizes to the leading edge, which is similar to Rap1-GTP and consistent with its involvement in regulation of adhesion at the anterior of moving cells. The localization of RapGAP1 in chemotaxing cells is mediated by F-actin and actin-bundling proteins cortexillins (Jeon et al., 2007b; Jeon et al., 2007a). Defects in spatial and temporal regulation of Rap1 activity and cell attachment at the leading edge in *rapGAP1* null cells or cells expressing RapGAP1 lead to defective chemotaxis. GFP-RapGAP1 overexpressing cells are unable to temporally and spatially regulate substratum attachments near the anterior of the cell immediately after pseudopod extension. In cells expressing GFP-RapGAP1, an extended anterior remains off of the substratum for a longer time, during which it randomly shifts direction relative to the chemoattractant gradient (Jeon et al., 2007a).

RapGAPB and RapGAP3 have been also identified as Rap1 GTPase-activating proteins in *Dictyostelium* and shown to be involved in the multicellular developmental process of *Dictyostelium*. RapGAPB is required for the sorting of different cell types during development, but not cell motility or chemotaxis. Defects in RapGAPB affect prestalk and

prespore cell adhesion in *Dictyostelium*, leading to abnormal morphogenesis of the multicellular organisms and misregulation of cell-type patterning during development (Parkinson et al., 2009). RapGAP3 mediates the deactivation of Rap1 during the late mound stage of development and plays an important role in regulation of cell sorting during apical tip formation by controlling cell-cell adhesion and cell migration. Direct measurement of cell motility within the multicellular organism mound shows that *rapGAP3* null cells have a reduced motility toward the apex, resulting in severely altered morphogenesis during development (Jeon et al., 2009).

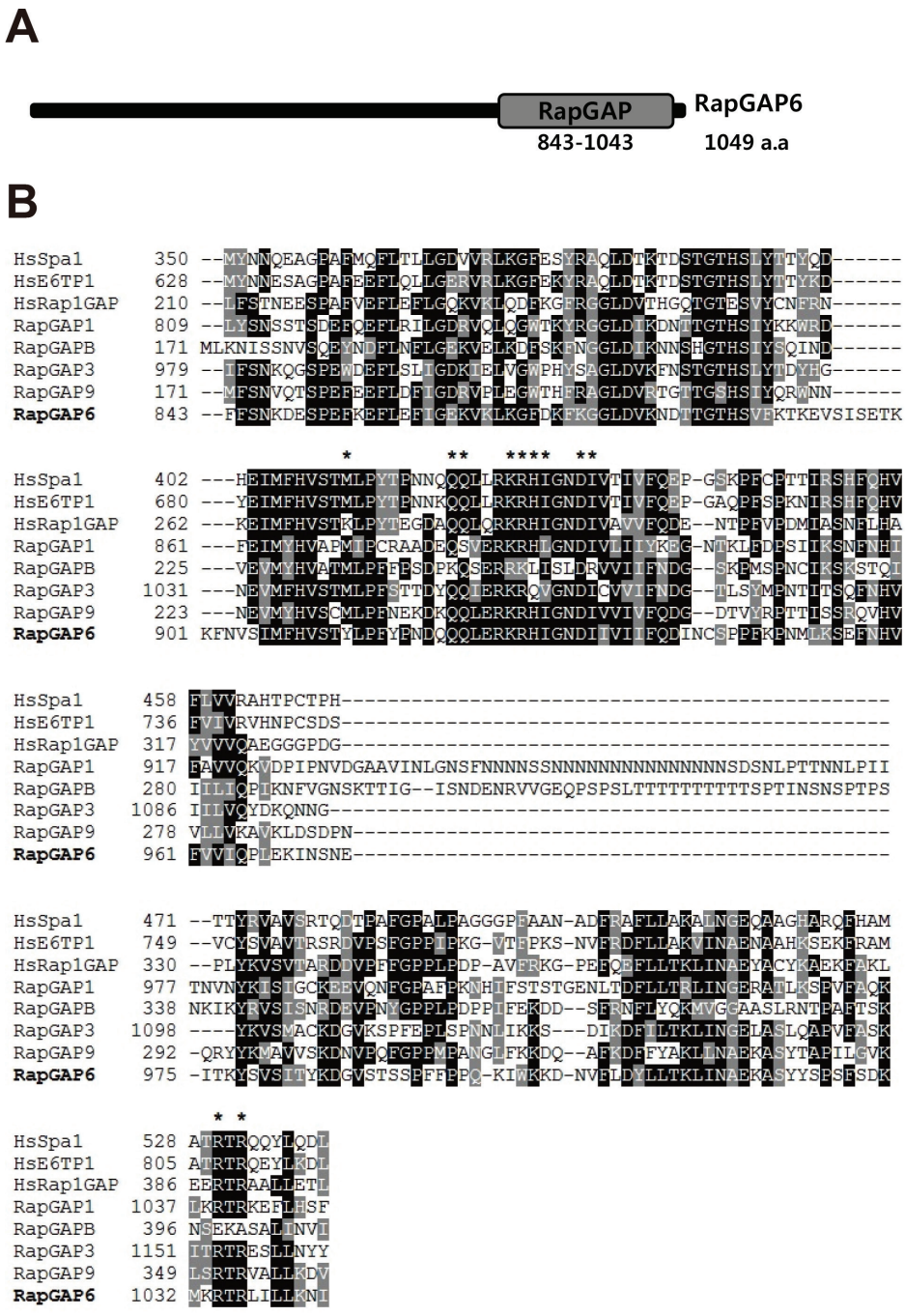
Recently RapGAP9 was identified as a putative specific GAP protein for Rap1 and required for proper development and morphogenetic processes (Mun et al., 2014). Loss of RapGAP9 resulted in an altered morphology of fruiting body with a shorter length of stalk and spore. RapGAP9 is involved in cell adhesion and multicellular development. Localization assay showed that RapGAP9 localized to the cell cortex and preferentially at the leading edge, in migrating cells. Upon chemoattractant stimulation, RapGAP9 was translocated transiently and rapidly to the cell cortex from the cytosol (Mun et al., 2014).

To further understand functions of Rap1, I have previously performed bioinformatic search for Rap1GAPs, and identified 10 open reading frames containing a putative RapGAP domain (Jeon et al., 2007b). The present study examines RapGAP6 (DDB0233725), one of the putative Rap1 GAPs which are expected to function as a Rap1 GAP protein. RapGAP6 is involved in morphogenesis, cell adhesion, and migration in *Dictyostelium*.

2. Results

1) Computer-based analysis of RapGAP6

To further characterize the RapGAP6 protein, I performed computer-based analyses. *Dictyostelium* RapGAP6 (DDB0233725) is composed of 1,049 amino acids (expected molecular mass 117 kDa) and contains a GAP domain at the C-terminal region (Fig. 1A). Multiple alignments of the GAP domain of RapGAP6 with those of other GAP proteins show that the RapGAP6 GAP domain is 46 % identical to human Rap1GAP. The GAP domain of RapGAP6 contains the catalytic Asn932 residue (Asn280 in HsRap1GAP) which is required for Rap1 GAP activity (Fig. 1B and 1C) (Brinkmann et al., 2002; Daumke et al., 2004). The phylogenetic trees show that RapGAP6 is related to the RapGAP family instead of the RasGAP family (Fig. 1D, RapGAP6 indicated by arrow). These results suggest that RapGAP6 functions as a Rap1-specific GAP protein.



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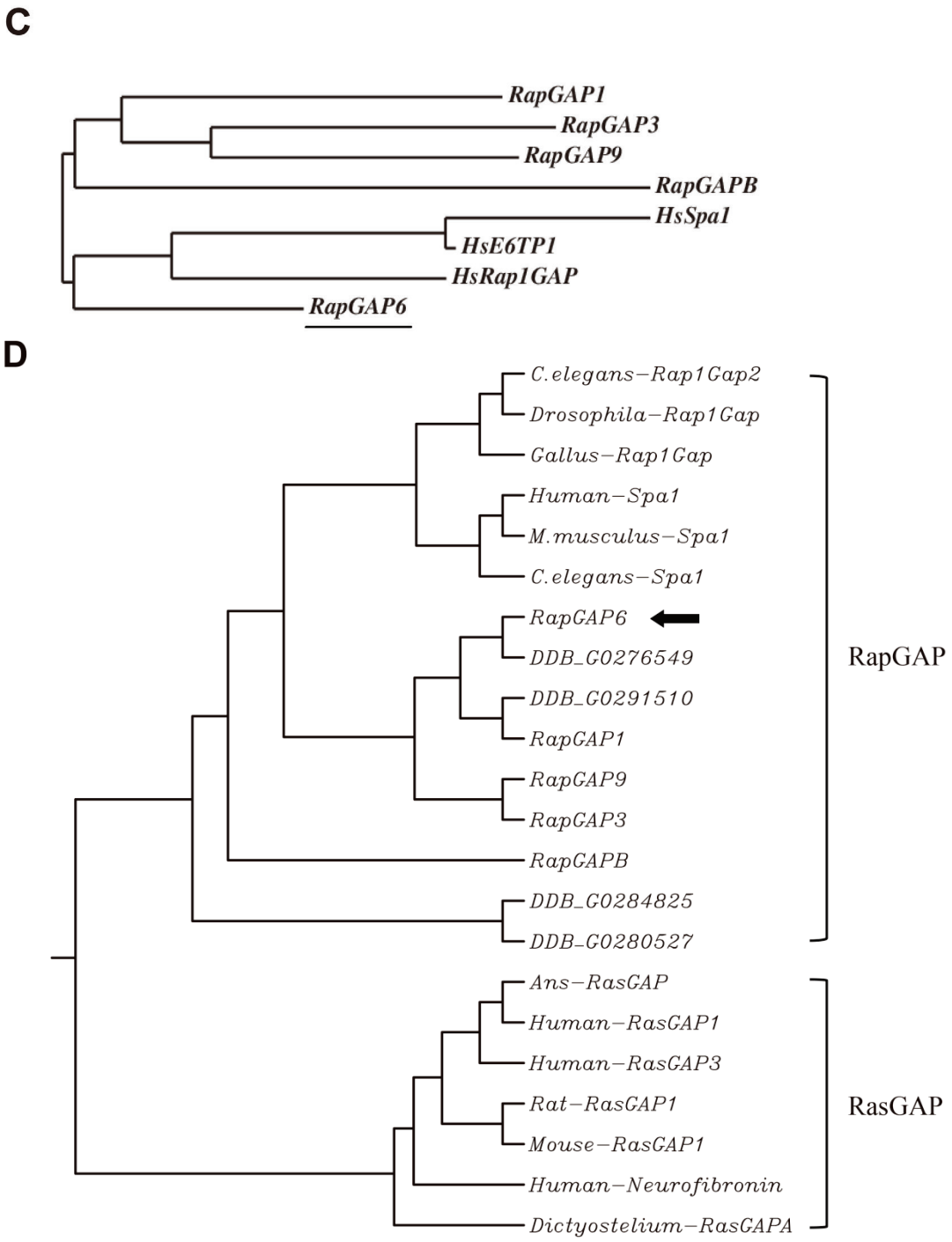

Fig. 1. (continued)

Fig. 1. Domain structure of RapGAP6 and multiple alignments of different RapGAPs and RasGAPs.

(A) Domain structure of RapGAP6. RapGAP6 contains RapGAP domain at the C-terminal region in *Dictyostelium*. (B) Multiple alignments of RapGAPs from diverse species. The amino acid sequence of a GAP domain of RapGAP6 was compared with those the following organisms. HsSpa1, *Homo sapiens* Spa1 (O60618); HsE6TP1, *H. sapiens* (Q9UNU4); HsRap1GAP, *H. sapiens* (P47736); DRapGAP1, *Dictyostelium discoideum* (dictyBaseID: DDB0233726); DRapGAPB, *D. discoideum* (dictyBaseID: DDB0233728); DRapGAP3, *D. discoideum* (dictyBaseID: DDB0229869); DRapGAP9, *D. discoideum* (dictyBaseID: DDB0233724); and DRapGAP6, *D. discoideum* (dictyBaseID: DDB0233725). These sequences are available at www.dictybase.org. Conserved residues were box-shaded, and asterisks indicate structurally or catalytically important amino acid residues (Daumke et al., 2004). (C) Phylogenetic tree analysis of the GAP domains from *Dictyostelium* and other species. RapGAP domain of RapGAP6 in *Dictyostelium* displays considerable sequence identicality to human Rap1GAP (46% identicality). (D) RapGAPs and RasGAPs domain phylogenetic tree: Human-Neurofibronin (P21359); Anus-RasGAP (B1AKC7); Human-RasGAP3 (Q14644); Human-RasGAP1 (O95294); Rat-RasGAP1 (P50904); Mouse-RasGAP1 (P50904); *Caenorhabditis elegans*-Rap1Gap2 (Q86RS9); Human-Spa1 (Q96FS4); *Gallus*-Rap1Gap (Q5ZMV8); *Mus musculus*-Spa1 (P46062); *C.elegans*-Spa1; *Drosophila*-Rap1Gap (O44090); *Dictyostelium*-RapGAP1 (dictyBaseID: DDBG0271734); RapGAPB (dictyBaseID: DDBG0282247); RapGAP3 (dictyBaseID: DDBG0271806); (dictyBaseID: DDBG0291510); (dictyBaseID: DDBG0276549); RapGAP9 (dictyBaseID: DDBG0284065); (dictyBaseID: DDBG0284825); (dictyBaseID: DDBG0280527); RapGAP6 (dictyBaseID: DDBG0283057). These sequences are available at www.dictybase.org

2) Confirmation of a *rapGAP6* knockout cells and overexpressing cells

To further understand the role of RapGAP6 in *Dictyostelium*, I prepared the *rapGAP6* null strains by homologous recombination. The *rapGAP6* knockout construct was prepared by inserting the bsr (blasticidin resistance) cassette into *rapGAP6* cDNA and used for a gene replacement in KAx-3 parental strains (Fig. 2A and 2B). Randomly selected clones were screened by PCR with three sets of primers (Fig. 2B and Table 1). Genomic DNA was extracted from wild-type and *rapGAP6* null cells. In the PCR with II+IV primers, which are located outside of the bsr cassette in the knockout construct, a band of 557 bp was observed in wild-type but that of *rapGAP6* null cells was 1,954 bp. PCR with another primer set, I+III, showed 557 bp in both wild-type and *rapGAP6* null cells. VI primer, which is located inside of the bsr cassette, showed a band of 607 bp in *rapGAP6* null cells (Fig. 2B). These results show that the *rapGAP6* gene was replaced with the *rapGAP6* knockout constructs in *rapGAP6* null cells.

RT-PCR was performed to determine whether *RapGAP6* gene is expressed in *rapGAP6* null cells. The cDNAs were synthesized by reverse transcription with total RNAs extracted from wild-type cells and *rapGAP6* null cells. In PCR using VII+VIII primers, there was no band in *rapGAP6* null cells, but wild-type showed a band of 687 bp (Fig. 2B). These results suggest that the *rapGAP6* is not expressed in *rapGAP6* null cells.

To examine the function of RapGAP6, cells overexpressing GFP-RapGAP6 fusion proteins were prepared. The expression of the protein was confirmed by Western blot with anti-GFP antibodies (Fig. 2C).

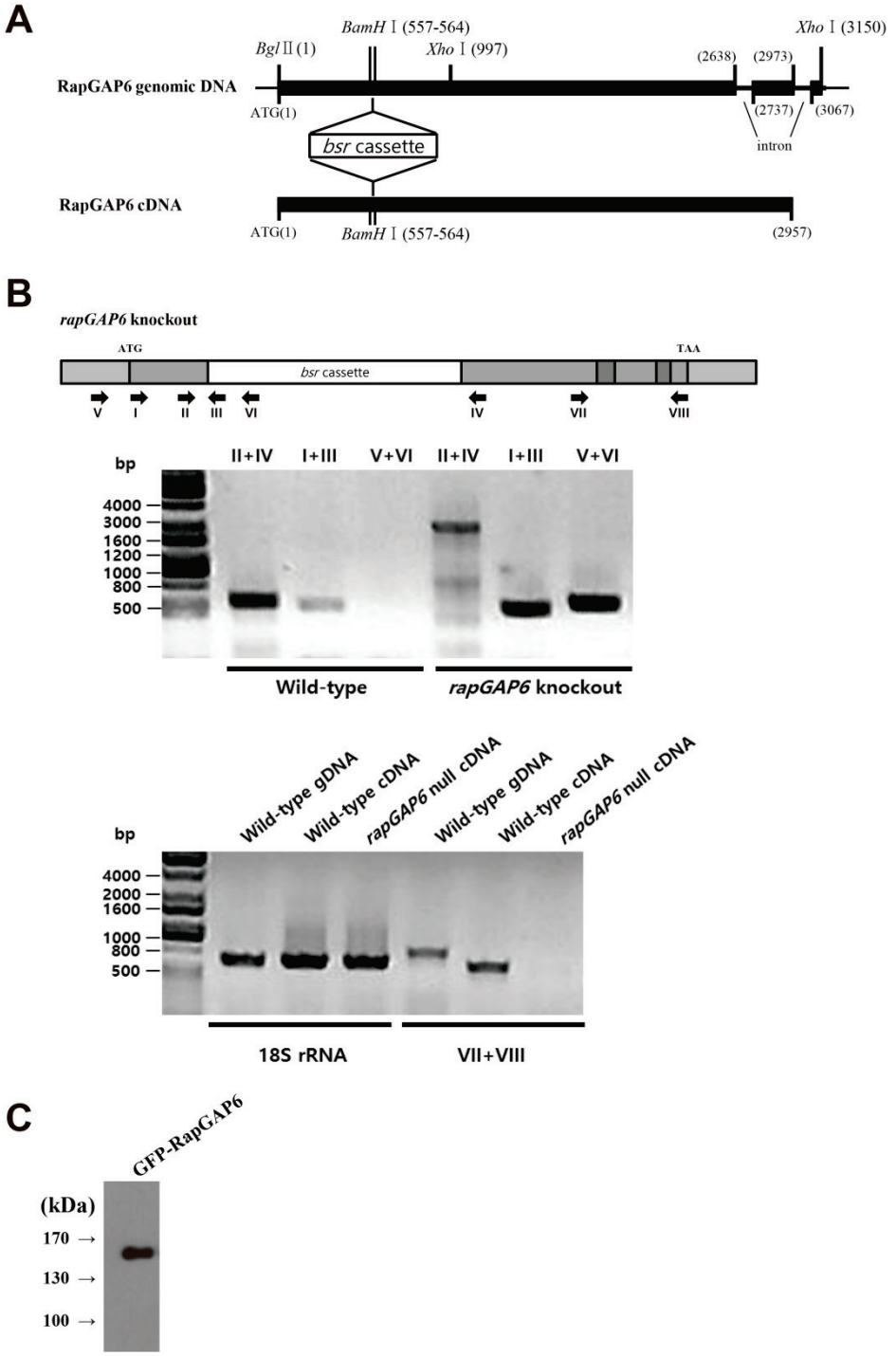

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Fig. 2. Confirmation of a *rapGAP6* knockout cells and overexpressing cells.

(A) Knock-out construct diagram. The location of the bsr cassette in RapGAP6 genomic DNA and cDNA. (B) Validation of *rapGAP6* null cells by PCR analysis. Primers are denoted by Roman numerals and arrows. The expression of *rapGAP6* in *rapGAP6* null cells was confirmed by RT-PCR. The 18S ribosomal RNA specific primers were used as an internal control. (C) Western blot analysis of GFP-RapGAP6. The *rapGAP6* null cells expressing GFP-RapGAP6 were performed by Western blot with an anti-GFP antibody.

3) RapGAP6 is involved in cell adhesion

To investigate the function of RapGAP6, I observed the phenotypes of wild-type, *rapGAP6* null cells, and RapGAP6 overexpressing cells. The *rapGAP6* null cells were more spread than wild-type cells (Fig. 3A).

RapGAP1 exhibits Rap1-specific GAP activity, which regulates cell attachment and cell spreading (Jeon et al., 2007b). *rapGAP6* null cells exhibited more filopodia, flatness, and spread compared with wild-type cells. The phenotype of *rapGAP6* null cells was weakly complemented by overexpression of RapGAP6. I measured the cell area using NIS-element and Image J program. This showed that the mean cell size of *rapGAP6* null cells was larger than wild-type cells with a high value of standard deviation (SD). In addition, frequency analysis of cell size shows that some of *rapGAP6* null cells were particularly flat and spread compared with wild-type cells (Fig. 3B).

To determine whether *rapGAP6* null cells have defects in cell adhesion, the strength of cell adhesion was examined by shaking culture and washing the plate, and counting the remained cells. *rapGAP6* null cells displayed a 1.4-fold increase in cell attachment compared with wild-type (Fig. 3C). *rapGAP6* null cell showed normal growth rates compared with wild-type cells (Fig. 3D). These results indicate that RapGAP6 is involved in cell morphogenesis and cell substratum adhesion.

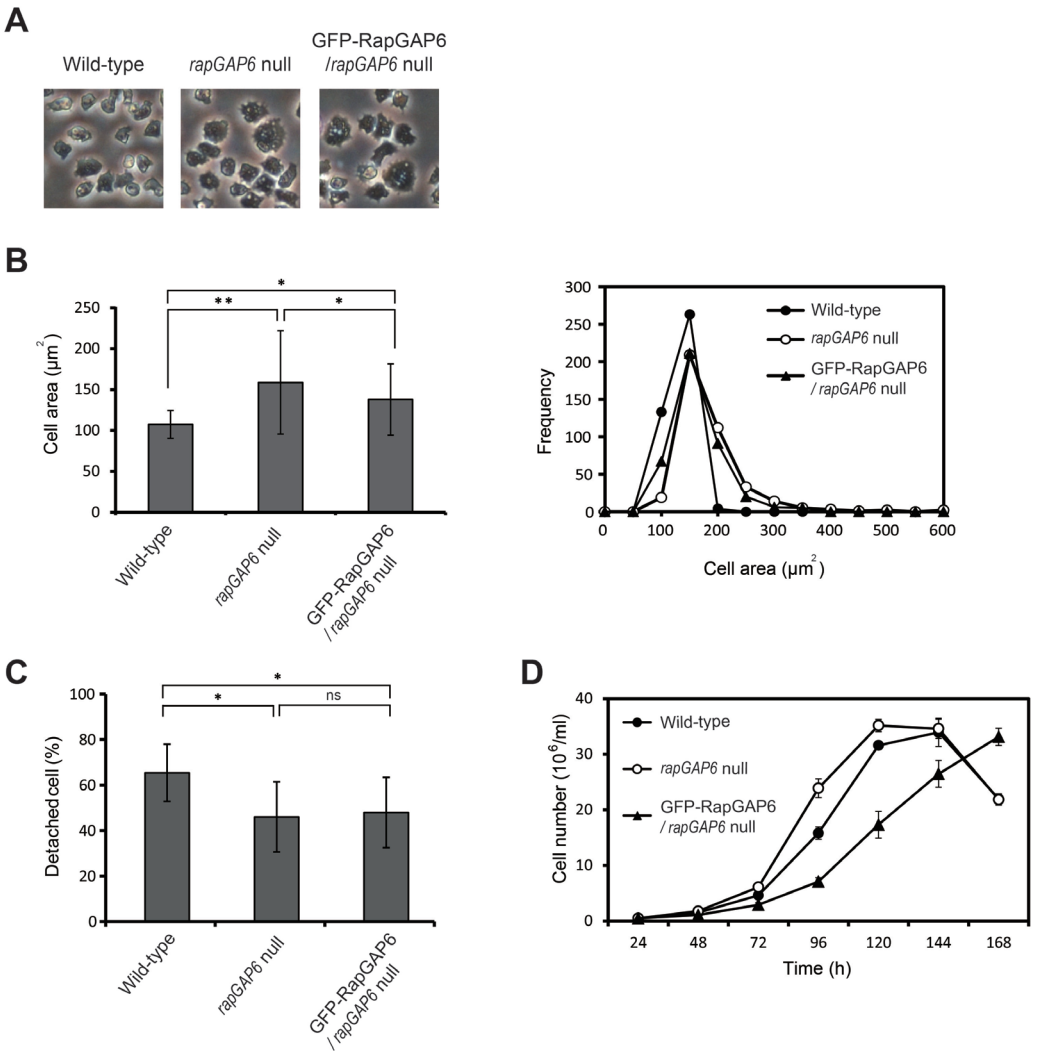


Fig. 3. (continued)

Fig. 3. Phenotypes of *rapGAP6* null and RapGAP6 overexpressing cells.

(A) Morphology of the vegetative cells. Wild-type cells, *rapGAP6* null cells, and GFP-RapGAP6 expressing cells were photographed. (B) Analysis of the cell area. The average values of the cell area were bar-graphed (left), and the frequency of the cell area is shown on the right. The results were expressed as the mean±SD (at least three independent times). Data were analyzed using Student's two tailed *t* test (*$p<0.05$ and **$p<0.01$ compared to the control). (C) Cell adhesion. Adhesion was measured by ratio of detached cells to the total number of cells. The values are the means±SD of three experiments (*$p<0.5$; ns, not significant compared to the control). (D) Growth rates of the cells.

4) RapGAP6 is required for directional sensing during chemotaxis

RapGAP1, a Rap1-specific GAP protein is required for regulation of cell adhesion by controlling Rap1 activity at the leading edge during migrating cells (Jeon et al., 2007b). In addition, the phenotypes of *rapGAP6* null cells are similar to cells expressing constitutively active Rap1.

To determine whether *rapGAP6* null cells are required for cell migration, I examined the chemotaxis using a cAMP chemoattractant and a Dunn chamber. I analyzed the cells by using NIS-element and Image J software. In response to cAMP stimulation, wild-type cells showed proper cell migration, and average speed was approximately 16 μm/min (Fig. 4B). The moving speed of *rapGAP6* null cells, was slightly higher than wild-type cells. However, the directionality was lower than wild-type cells (Fig. 4). These data suggest that RapGAP6 is required for proper cell migration and involved in directional sensing during chemotaxis.

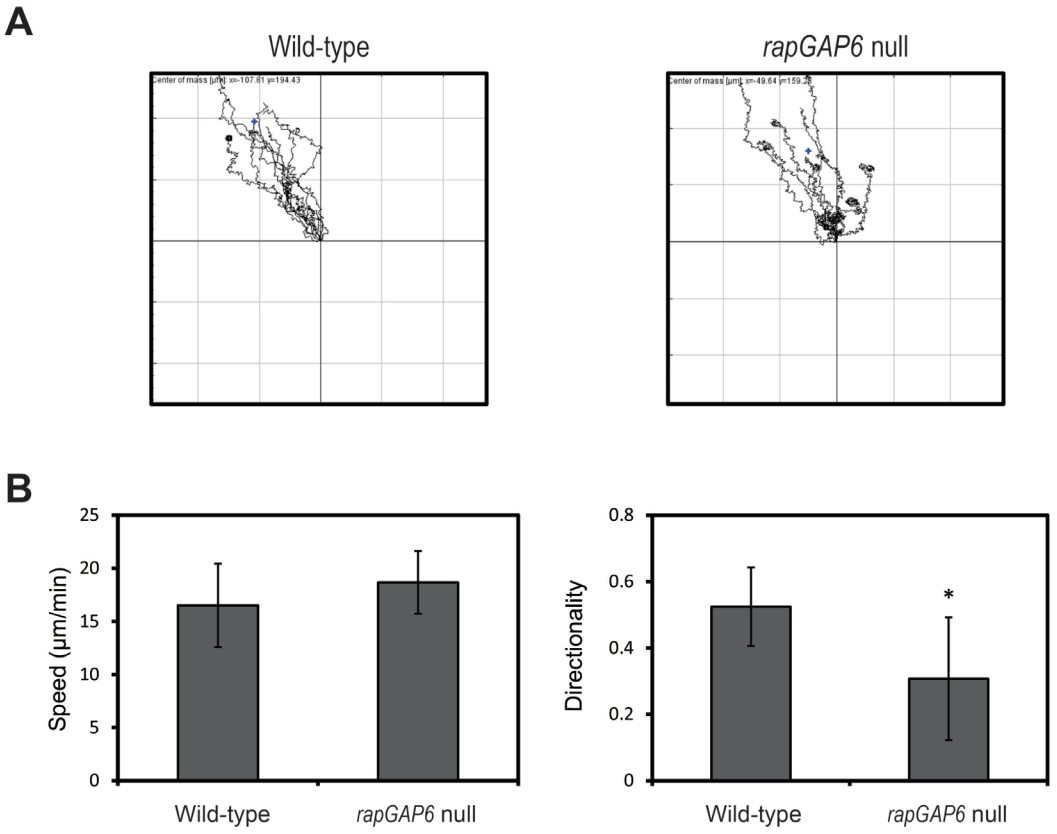


Fig. 4. Chemotaxis of *rapGAP6* null cells.

Aggregation competent cells from wild-type and *rapGAP6* null cells were placed in a Dunn chamber, and cells toward cAMP chemoattractant gradient were recorded by time lapse photography for 30 min at 6 sec intervals. (A) Trajectories of cells in Dunn chamber. Migrating cells were tracked with Image J program. Each line represents the track of a single cell chemotaxing toward 150 μM cAMP. (B) Speed and directionality during cell migration. Speed indicates the speed of the cell's movement along the total path. Directionality is a measure of how straight the cells move. Cells moving in a straight line have a directionality of 1.0. It is calculated by the distance between the start and finish. The values are the means±SD (*$p<0.05$ compared to the control).

5) RapGAP6 is dispensable to development

A developmental process is induced during starvation with cells plated on a non-nutrient agar plate. To investigate the role of RapGAP6 during development, I prepared the wild-type cells, *rapGAP6* null cells, and RapGAP6 overexpressing cells and performed development assay (Fig. 5). *rapGAP6* null cells aggregated normally to form a mound at ~8 h, with timing and morphology similar to wild-type cells. In addition, expression of GFP-RapGAP6 in *rapGAP6* null cells was similar to that of wild-type cells in developmental stage. These results show that RapGAP6 does not affect development.

Additionally, RapGAP3 is required for proper development, *rapGAP3* null cells aggregate normally to form mounds at ~8 h, but disaggregate at ~12 h and form ring-like structures (Jeon et al., 2009). To determine whether RapGAP6 is involved in the tip forming stage during development, RapGAP6 was expressed in *rapGAP3* null cells, and the phenotypes of *rapGAP3* null cells expressing RapGAP6 were compared with those of wild-type cells and *rapGAP3* null cells (Fig. 5). The phenotypes observed in *rapGAP3* null cells were not complemented by overexpression of RapGAP6, indicating that the function of RapGAP3 is not overlapped with that of RapGAP6.

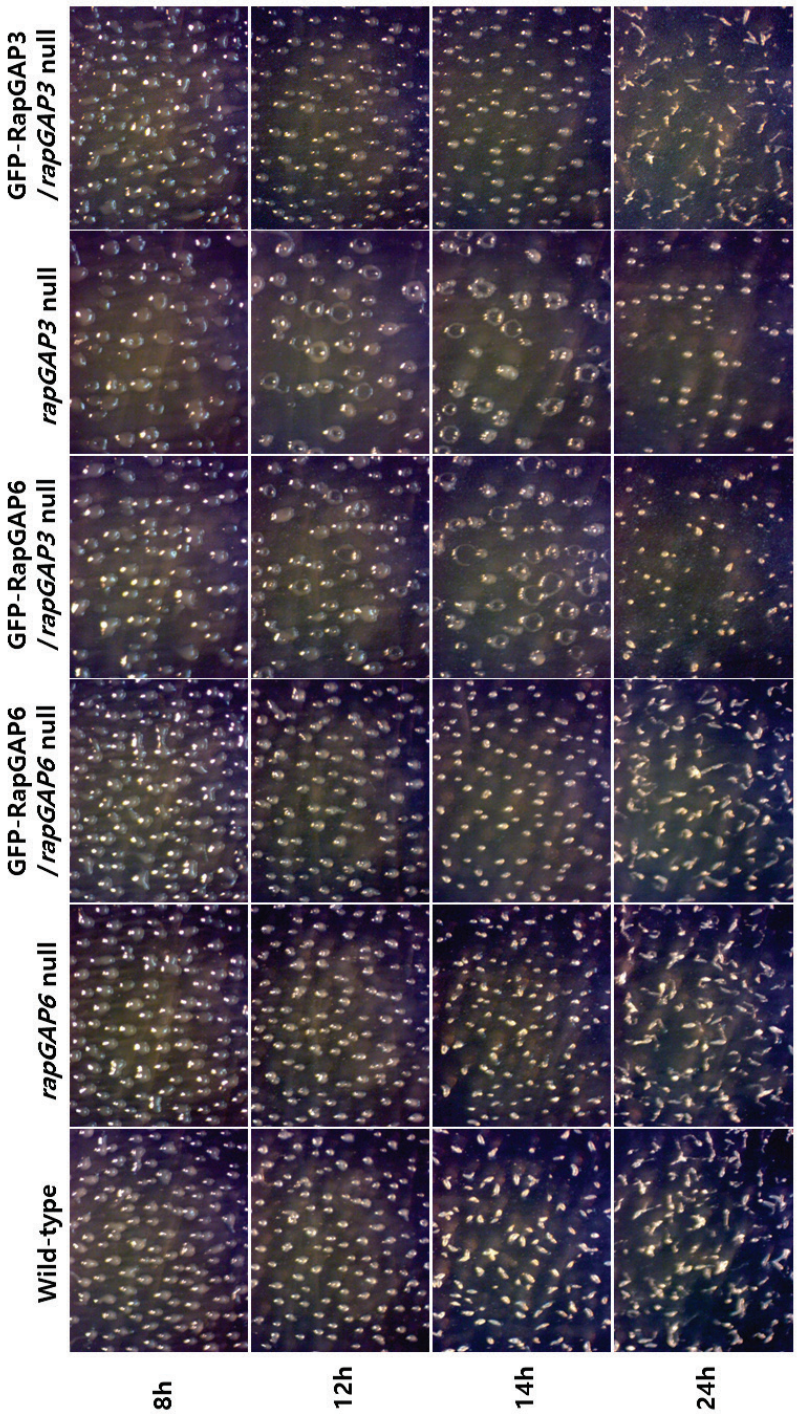


Fig. 5. (continued)

Fig. 5. Development of *rapGAP6* null cells and complementation experiments.

Developmental phenotypes of *rapGAP6* null cells and complementation of the phenotype of *rapGAP3* null cells by RapGAP6. Wild-type cells, *rapGAP6* null cells, GFP-RapGAP6/*rapGAP6* null cells, *rapGAP3* null cells, GFP-RapGAP6/*rapGA3* null cells, GFP-RapGAP3/*rapGA6* null cells, and GFP-RapGAP3/*rapGA3* null cells. Vegetative cells were washed with Na/K buffer and developed on the non-nutrient agar plates. Photographs were taken at the indicated times after plating.

6) RapGAP6 is localized in the cytosolic vesicles

To determine the localization of the RapGAP6 proteins, I prepared GFP-RapGAP6 expressing cells and observed the localization of the proteins in the cells. GFP-RapGAP6 was found in the vesicles in the cytosol (Fig. 6B). To further understand the localization mechanism of RapGAP6 in the vesicles, I prepared cells expressing GFP-GAP or GFP-ΔGAP truncated proteins and examined the localizations of the proteins (Fig. 6A). These proteins indicating GFP-GAP and GFP-ΔGAP were observed in the cytosol not in the vesicles (Fig. 6B). These results suggest that the full-length RapGAP6 is required for localization in the vesicles. Then, for comparison other RapGAP proteins, I prepared the cell lines of GFP-RapGAP6 in *rapGAP1* null cells, *rapGAP3* null cells, and *rapGAP9* null cells. GFP-RapGAP6 was observed in the vesicles in *rapGAP1* and *rapGAP9* null cells, suggesting that the vesicle localization of RapGAP6 is not affected by RapGAP1 and RapGAP9 (Fig. 6C).

To further understand the mechanism behind the localization of vesicles in cytosol, I examined the phagocytosis plaque assay. On bacterial lawns, wild-type, *rapGAP6* null cells and GFP-RapGAP6 expression in *rapGAP6* null cells permitted phagocytosis and showed clear plaques (Fig. 6D). These results suggest that RapGAP6 is unlikely to be involved in phagocytosis. However, further experiments are needed to see if RapGAP6 plays some roles in phagocytosis.

To determine whether RapGAP6 has GAP activity to Rap1, I used a pull-down assay using the human RalGDS Rap1-GTP-biding domain. *rapGAP6* null cells seem to have slightly higher level of Rap1-GTP compared to wild-type cells (Fig. 6E). However, further experiments are required to see if RapGAP6 has a GAP activity specific to Rap1.

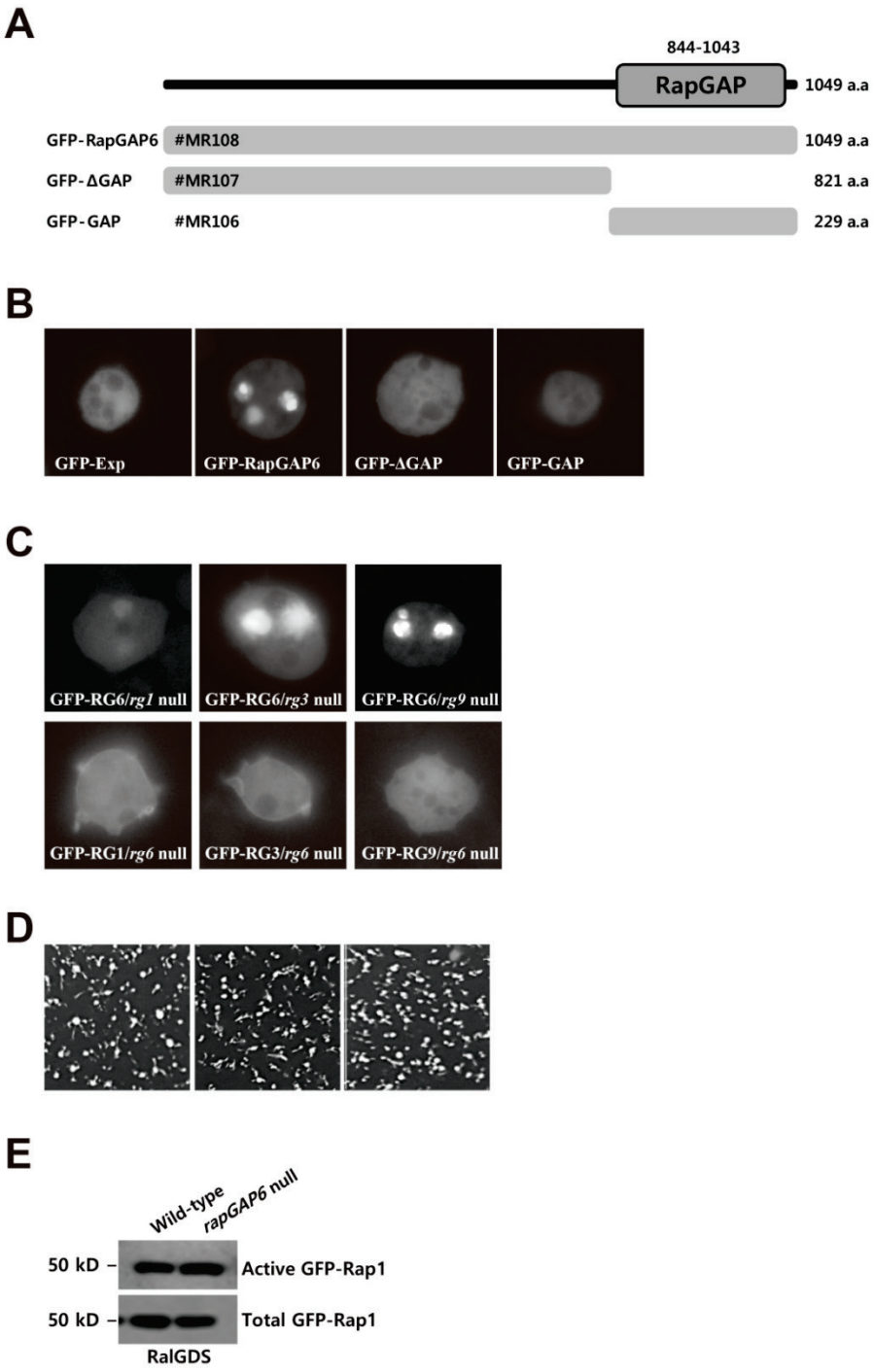


Fig. 6. (continued)

Fig. 6. Localization of GFP-RapGAP and plaque assay on bacterial lawns.

(A) Diagram of the GAP domain truncated fragments (B) Localization of GFP-RapGAP6, GFP-ΔGAP, and GFP-GAP in *rapGAP6* null cells. Fluorescent images of GFP fusion proteins in *rapGAP6* null cells. (C) Localization of RapGAP1, RapGAP3 and RapGAP9. (D) Development of wild-type, rapGAP6 null, and GFP-RapGAP6 in *rapGAP6* null cells on bacterial lawns on SM plate. (E) Rap1 pull-down assay of *rapGAP6* null cells. Lysates from GFP-expressing wild-type cells and *rapGAP6* null cells were used in the pull-down assay using GST-RalGDS-RBD. The samples were subjected to Western blot using an anti-GFP antibody.

3. Discussion

Rap1-specific GAP proteins including RapGAP1, RapGAPB, and RapGAP9 have been previously identified and characterized. RapGAP6 was predicted one of the putative RapGAP proteins by computer-based analyses. In this study, I have characterized RapGAP6 and found that RapGAP6 is involved in cell morphogenesis, adhesion, and migration. The loss of RapGAP6 resulted in severe defects in cell morphology and cell migration, but not in development. *rapGAP6* null cells showed slightly strong adhesion and flattened cell morphology. RapGAP6 was localized in the cytosolic vesicles, suggesting that RapGAP6 might function in the process of phagocytosis.

RapGAP1 is required for the regulation of cell adhesion by controlling Rap1 activity at the leading edge of chemotaxing cells. In chemotaxing cells, RapGAP1 preferentially localizes to the leading edge, mediated by F-actin and actin-bundling proteins called cortexillins (Jeon et al., 2007b; Jeon et al., 2007a). RapGAP6 has defect in cell migration induced by chemoattractants. Some of the cells move approximately normally, but in the middle of aggregation, cells move around the start position. RapGAP6 is required for chemotaxis. RapGAP1 plays an important role in chemotaxis, but not in development. RapGAPB and RapGAP3 are involved in the multicellular developmental process of *Dictyostelium*. RapGAPB is required for the correct sorting behavior of different cell types during development, but not cell motility or chemotaxis (Parkinson et al., 2009). RapGAP3 mediates the deactivation of Rap1 during the late mound stage of development and plays an important role in regulation of cell sorting during apical tip formation by controlling cell-cell adhesion and cell migration (Jeon et al., 2009). RapGAP6 is not involved in development. In addition, RapGAP6 was not complemented with RapGAP3 during development. These results show that RapGAP6 does not play an important role in development, but it does in

cell adhesion and cell morphogenesis.

RAPGAP9 was localized in the cell cortex preferentially at the leading edge, in migrating cells (Mun et al., 2014). On the other hand, full-length RapGAP6 is required for localization in the vesicles. The vesicle localization of RapGAP6 is not affected by RapGAP1, RapGAP3, and RapGAP9. These results suggest that RapGAP6 is unlikely to be involved in phagocytosis. However, further experiments are needed to determine if RapGAP6 plays roles in phagocytosis.

This study identified and characterized RapGAP6 as one of the Rap GAP proteins in *Dictyostelium discoideum*. RapGAP6 controls cell adhesion and morphogenesis. RapGAP6 is involved in directional sensing during chemotaxis, but in defects during development. RapGAP6 is localized in the cytosolic vesicles and is unlikely to be involved in phagocytosis. *rapGAP6* null cells seem to have a slightly higher level of Rap1-GTP compared with wild-type cells. However, further experiments are required to determine if RapGAP6 has GAP activity specific to Rap1.

Part III. Characterization of a Rap1 downstream effector FrmB in *Dictyostelium*

1. Introduction

The globular four-point-one, ezrin, radixin and moesin (FERM) domain is composed of three subdomains (F1, F2 and F3) like a clover leaf (Bretscher et al., 2002). FERM domain proteins regulate cell-substratum adhesion and focal adhesions during cell migration in mammalian cells (Lo, 2006). In *Dictyostelium*, there are six proteins that contain FERM domain, TalinA, TalinB, MyosinVII, FrmA, FrmB and FrmC (enlazin) (Niewohner et al., 1997; Octtaviani et al., 2006; Tsujioka et al., 1999; Tuxworth et al., 2001). TalinA and TalinB regulate cell-substratum adhesion, and localize to the leading edge of cells during cell migration (Hibi et al., 2004; Niewohner et al., 1997; Tsujioka et al., 2004). MyosinVII and FrmC reduce cell-substratum adhesion (Octtaviani et al., 2006; Tuxworth et al., 2001). The talin-like FERM domains of FrmA are required for correct cell-substratum adhesion. FrmA regulates the temporal and spatial control of talinA and paxillin at cell-substratum adhesion sites, which in turn controls adhesion and motility (Patel et al., 2008). FrmA is required in regulating cell-cell adhesion, multi-cellular development in *Dictyostelium* (Patel and Brunton, 2009).

The MRL family proteins contains a Ras association (RA) domain that interacts with Rap1-GTP and stimulates integrin-mediated cell adhesion and cell spreading (Boettner and Van Aelst, 2009; Krause et al., 2004; Lee et al., 2009). Krit1, another Rap1 effector, contains an FERM domain and controls endothelial cell-cell junctions (Glading et al., 2007). Arap3 is a RhoGAP containing five PH domains and an RA domain that interacts with Rap1 and

affects PDGF-induced lamellipodia formation (Krugmann et al., 2006).

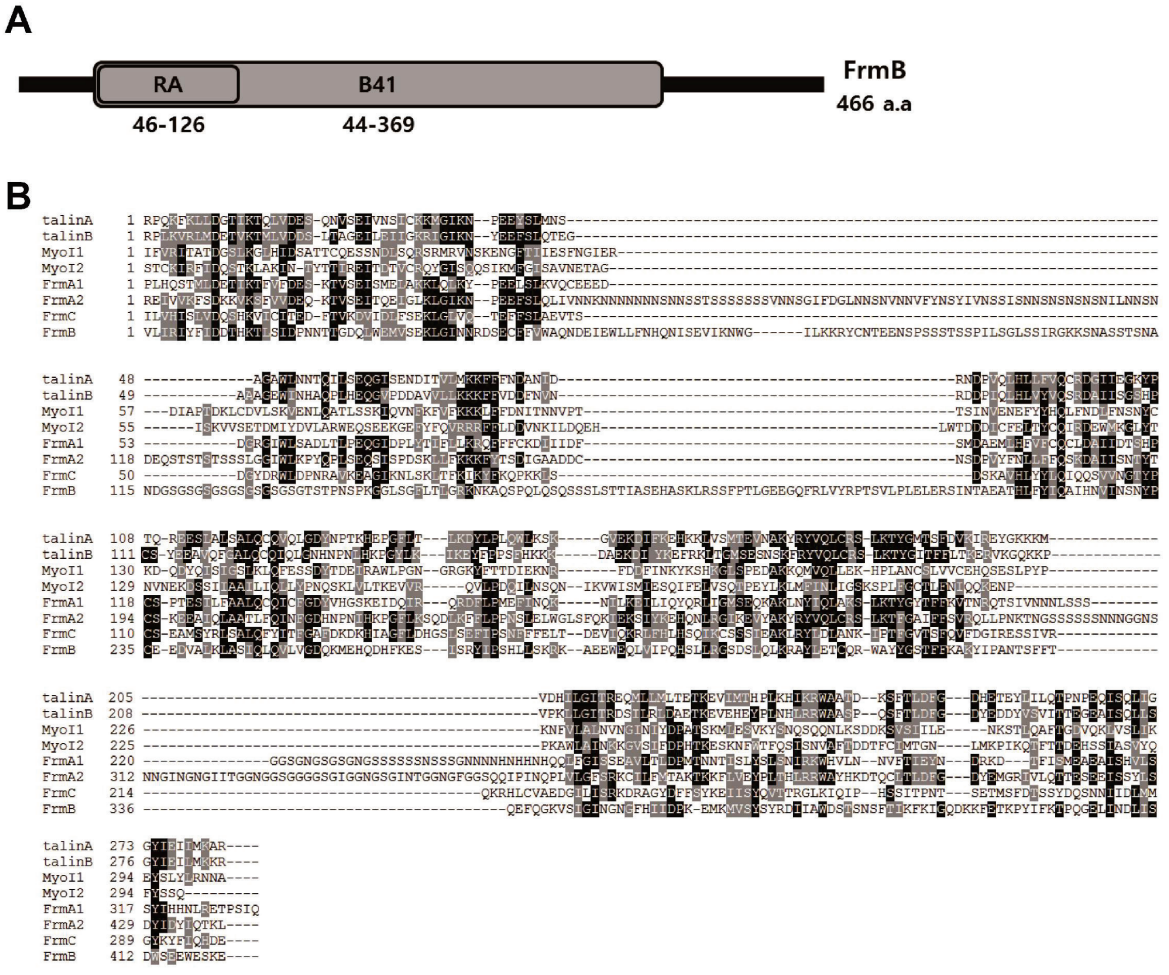
To further understand functions of other Rap1 downstream effectors, I conducted a database search for Rap1-GTP binding domains, FERM and RA domains, and identified six FERM domain containing proteins. Here, I investigated that FrmB (DDB0233516) and found that FrmB is involved in cell adhesion and development in *Dictyostelium*.

2. Results

1) Computer-based analysis of FrmB

To further investigate the RA containing proteins, I used a bioinformatics approach and identified the FERM domain containing proteins. I examined the computer-based analysis and identified six FERM proteins in *Dictyostelium.* I studied one of these proteins, referred to as FrmB (DDB0233516). *Dictyostelium* FrmB is composed of 478 amino acids (expected molecular mass 53kDa) and contains FERM, B41, and RA domains (Fig. 1A).

To understand the role of FERM domain proteins in FrmB, they were compared with other FERM domain proteins in *Dictyostelium*. The sequences were aligned by using the multiple sequence alignment program CLUSTAL W (Fig. 1B and 1D). The B41 domain of FrmB was related to the FERM containing RA proteins. I aligned the RA domain with human RalGDS and RIAM. FrmB had a well conserved RA domain. The phylogenetic trees show that FrmB is closest to Talin A (Fig. 1C). These results suggest that FrmB is expected to function as a Rap1 downstream effector.



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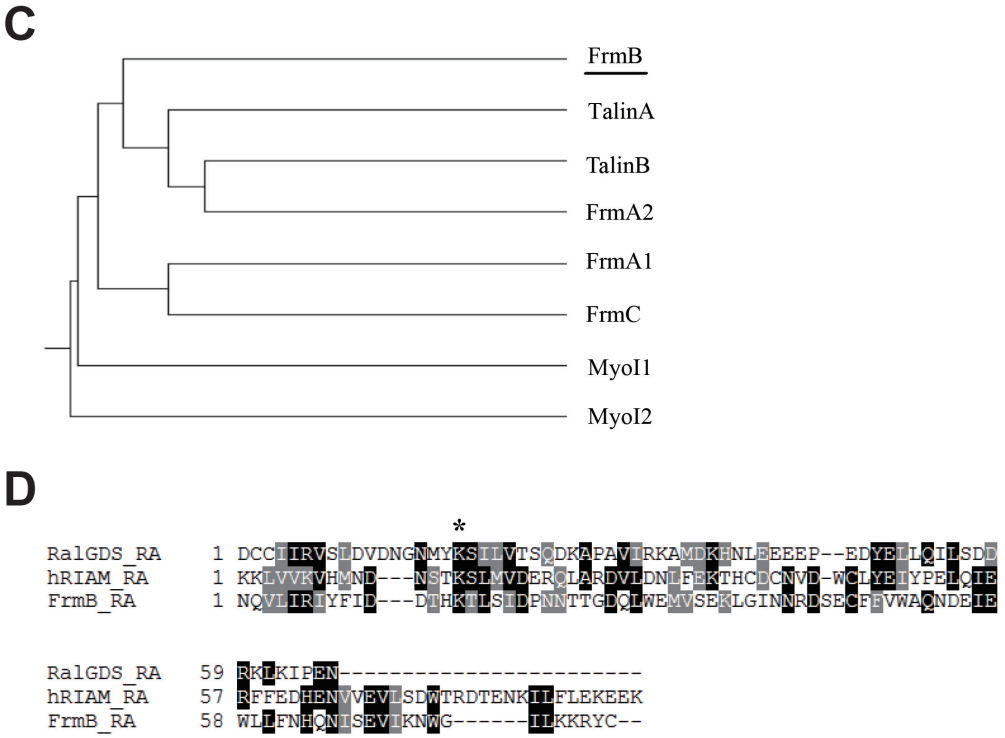

Fig. 1. (continued)

Fig. 1. Domain structure and multiple alignments of FrmB.

(A) Domain structure of FrmB. FrmB contains a B41 domain containing RA domain in *Dictyostelium*. (B) Multiple alignments of FERM domain in *Dictyostelium*: DdtalinA, DdtalinB, DdMyoI1, DdMyoI2, DdFermA1, DdFermA2, DdFrmB, DdFrmC. These sequences are available at www.dictybase.org. (C) Phylogenetic trees with FERM domains in *Dictyostelium*. (D) Multiple alignments of RA domain with human RalGDS, human RIAM, and *Dictyostelium* FrmB.

2) Confirmation of *frmB* knockout cells and overexpressing cells

To further understand the role of FrmB in *Dictyostelium*, I prepared the *frmB* null strains by homologous recombination. The *frmB* knockout construct was prepared by inserting the bsr (blasticidin resistance) cassette into *frmB* cDNA and used for a gene replacement in KAx-3 parental strains (Fig. 2A and 2B). Randomly selected clones were screened by PCR with three sets of primers (Fig. 2B and Table 1.). In addition, genomic DNA was extracted from wild-type and *frmB* null cells. In the PCR with II+IV primers, which are located outside of the bsr cassette in the knockout construct, a band of 605 bp was observed in wild-type whereas a band of 1640 bp was found in *frmB* null cells. In the PCR using another primer set (II+V), which is located outside of the bsr cassette, a band of in 833 bp and 1868 bp was observed in wild-type and *rapGAP6* null cells, respectively. When the primer III, was used in the PCR, a band of which is located inside of the bsr cassette, 636 bp were amplified in both wild-type and *frmB* null cells (Fig. 2B). These results indicate that the *frmB* gene was replaced with the *frmB* knockout constructs in *frmB* null cells.

To examine the function of FrmB, overexpressing cells of GFP-FrmB fusion proteins were prepared. The expression of the protein was confirmed by Western blot with anti-GFP antibodies (Fig. 2C).

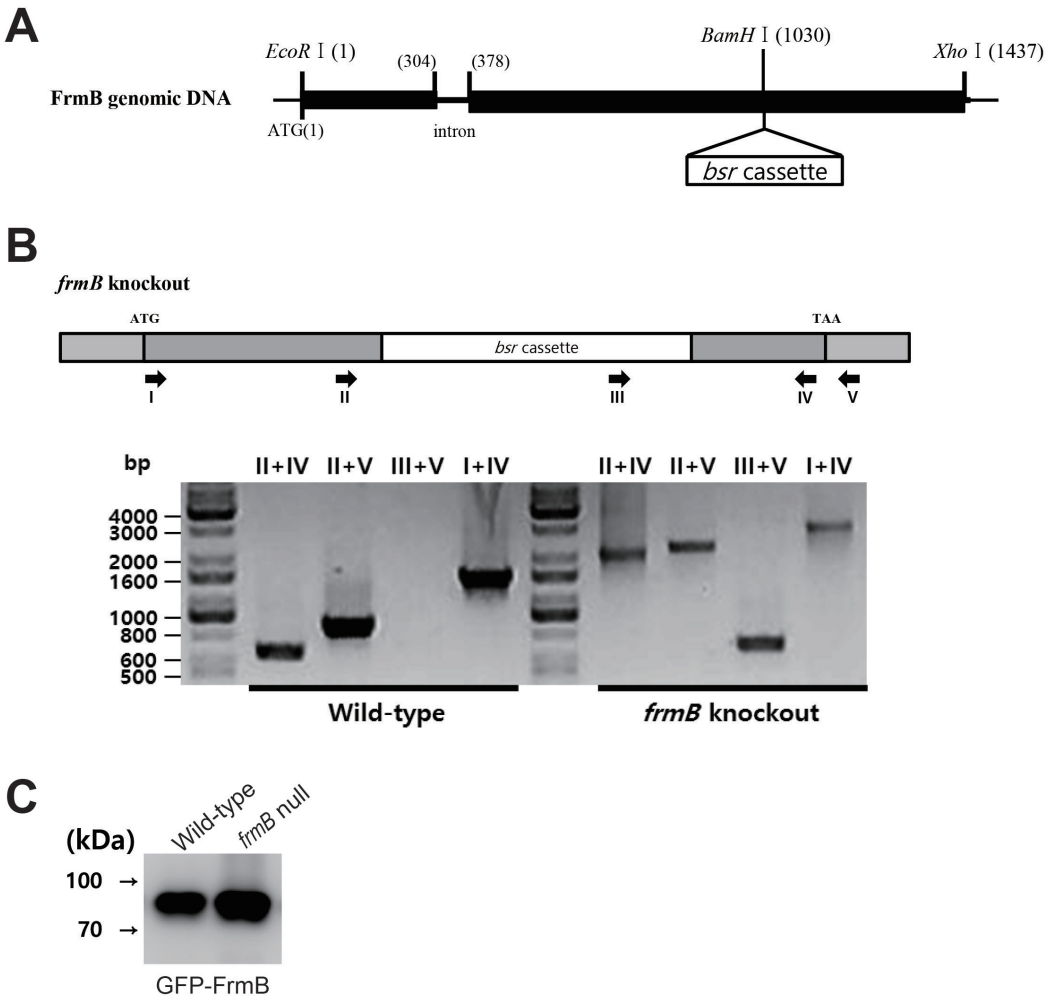

Fig. 2. Confirmation of *frmB* knockout cells and overexpressing cells.

(A) Knock-out construct diagram. The location of the bsr cassette in FrmB genomic DNA is illustrated. (B) Validation of *frmB* null cells by PCR analysis. Primers are denoted by Roman numerals and arrows. (C) Western blot analysis of GFP-FrmB in wild-type and *frmB* null cells. GFP-FrmB overexpressing cells were performed by Western blot with an anti-GFP antibody.

3) FrmB plays an important role in cell adhesion

To investigate the function of FrmB in cells, I examined the phenotypes of *frmB* null cells and FrmB overexpressing cells. The *frmB* null cells were slightly more spread and had more filopodia than wild-type cells (Fig. 3A). The phenotype of *frmB* null cells was complemented by introduction of FrmB into the *frmB* null cells. I measured the cell area using NIS-element and Image J program there was no significant difference among the cells (Fig. 3B). These results suggest that the *frmB* null cell is not important in cell morphogenesis.

To determine whether FrmB plays some roles in cell adhesion, the strength of cell adhesion of the *frmB* null cells was examined by shaking the cell-culture plate and washing the plate, and counting the number of the detached cells. *frmB* null cells displayed an 1.7 fold decreased cell adhesion compared to wild-type cells (Fig. 3C). *frmB* null cells exhibited weaker adhesion than wild-type cells. These results show that FrmB plays an important role in cell adhesion.

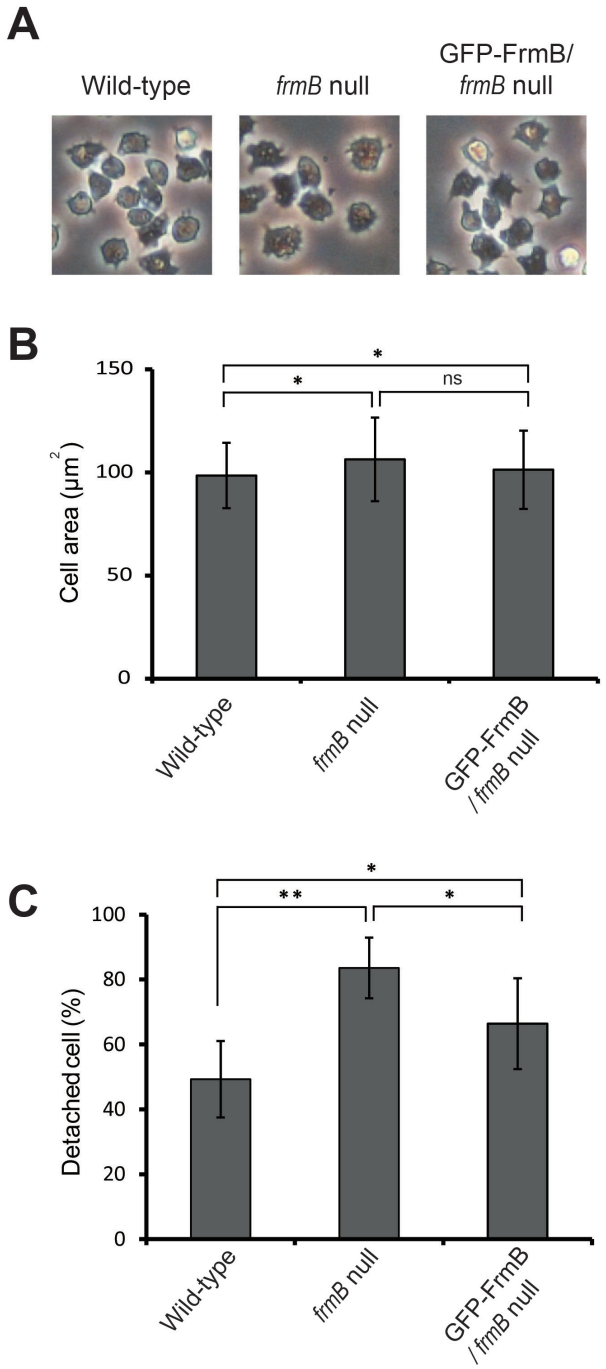

Fig. 3. (continued)

Fig. 3. Phenotype of *frmB* null cells and overexpressing cells.

(A) Morphology of the vegetative cells. Wild-type, *frmB* null cells, and *frmB* null cells expressing null cells were photographed. (B) Analysis of the cell area. The values are the means±SD of three experiments (*$p<0.5$; ns, not significant compared to the control). (C) Cell Adhesion. Adhesion was measured by ratio of detached cells to the total number of cells. The values are the means±SD of three experiments (*$p<0.5$ and **$p<0.01$ compared to the control).

4) FrmB is required for proper cell migration

To determine whether FrmB is required for cell migration, I examined the chemotaxis by a cAMP chemoattractant using a Dunn chamber. I analyzed the migrating cells using NIS-element and Image J program. In response to cAMP stimulation, wild-type cells showed proper cell migration with an average speed of approximately 16 μm/min (Fig. 4B). In *frmB* null cells, the migrating cell speed was lower than that in wild-type cells and the directionality was similar to wild-type cells and had high standard deviation. These data suggest that FrmB is required for proper cell migration.

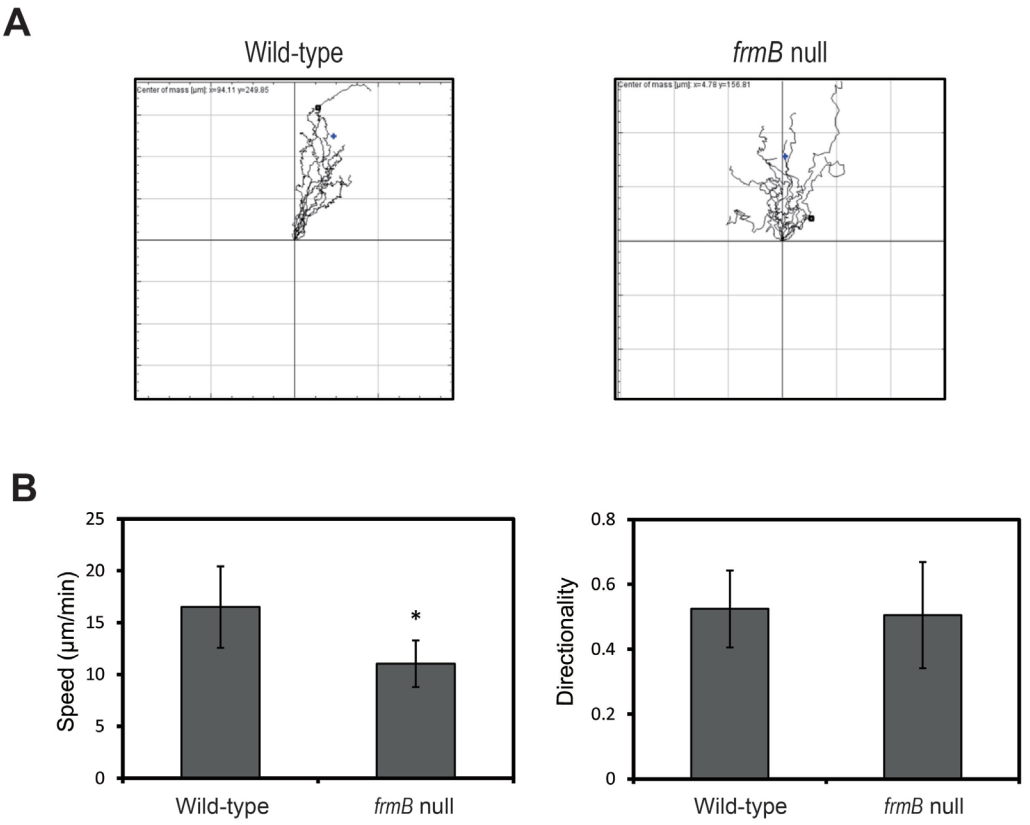

Fig. 4. Chemotaxis of *frmB* null cells.

Aggregation competent cells from wild-type and *frmB* null cells were placed in a Dunn chamber, and cells toward cAMP chemoattractant were recorded by time-lapse photography for 30 min at 6 sec intervals. (A) Trajectories of cells in Dunn chamber. Migrating cells were tracked with Image J program. Each line represents the track a single cell chemotaxing toward 150 μM cAMP. (B) Speed and directionality during cell migration. Speed indicates the speed of the cell's movement along the total path. Directionality is a measure of how straight the cells move. Cells moving in a straight line have a directionality of 1.0. It is calculated by the distance between the start and finish. The values are the means±SD (*$p<0.05$ compared to the control).

5) FrmB plays an important role in development

To investigate the role of FrmB during development, I prepared wild type, *frmB* null, and FrmB overexpressing cells and performed development assay (Fig. 5A). *frmB* null cells aggregated normally to form a mound at ~8 h, with timing and morphology similar to wild-type cells. The FrmB overexpressing cells were similar to wild-type in development. When the expression level of FrmB was increased by incubating the cells in the presents of high level of antibiotics (G10 and G50), the cells expressing FrmB showed no aggregation and no further progress of development (Fig. 5B and 5C). These results suggest that FrmB is not necessarilly required for proper development, but high level of FrmB inhibits aggregation of the cells during development.

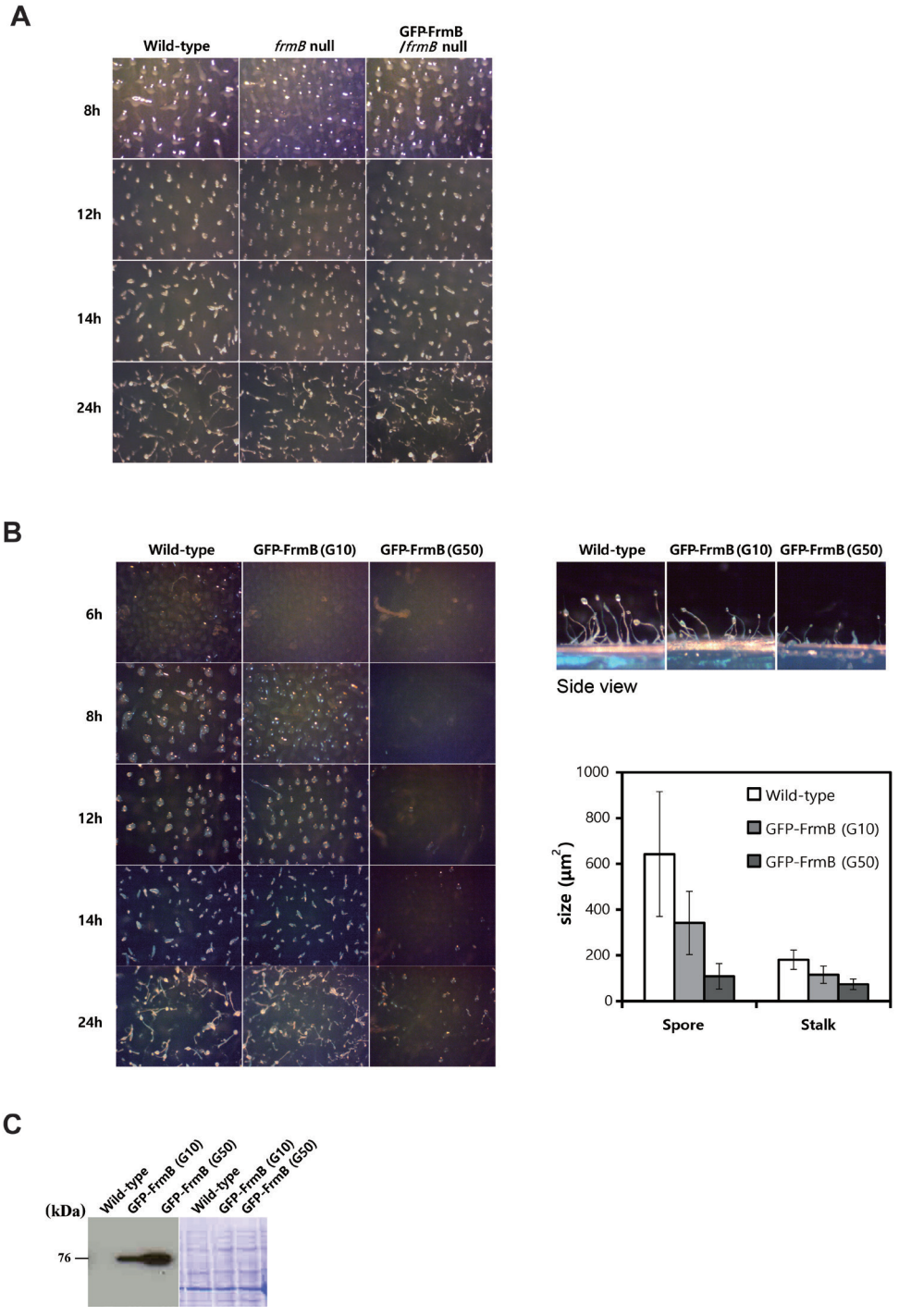

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Fig. 5. Development of *frmB* null cells, overexpressing cells, and GFP-FrmB.

(A) Developmental phenotypes of wild-type, *frmB* null cells, and GFP-FrmB/*frmB* null cells. Vegetative cells were washed with Na/K buffer and plated on non-nutrient agar plates. Photographs were taken at the represent time after plating. (B) Development of GFP-FrmB (G10 and G50). Vegetative cells were washed with Na/K buffer and plated on non-nutrient agar plates. Photographs were taken at 8, 12, 16, and 24 h after plating from a side view. (C) Western blot analysis of GFP-FrmB G10 and G50 with an anti-GFP antibody.

6) FrmB is localized in the cytosol

To determine the localization of the FrmB proteins in the cells, I prepared the GFP-tagged FrmB expressing cells. GFP-FrmB was found in the cytosol (Fig. 6A and 6B). The result shows that FrmB is localized in the cytosol which may function in the cytoplasm.

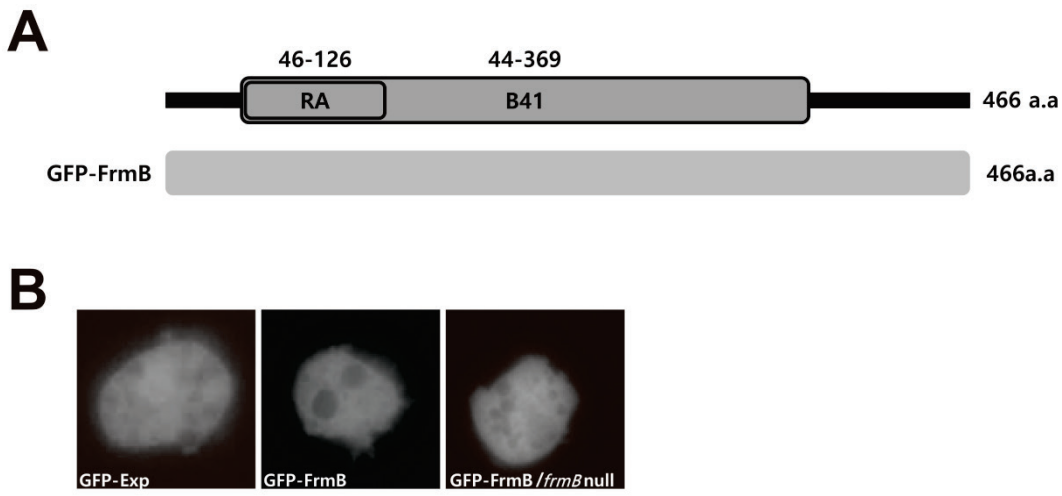

Fig. 6. Localization of GFP-FrmB

(A) Diagram of FrmB (B) Fluorescent images of GFP fusion proteins. Localization of GFP-Exp, GFP-FrmB in wild-type cells, and GFP-FrmB in *frmB* null cells.

3. Discussion

I investigated the function of FrmB, which might be a Rap1 downstream effector. FrmB has a B41 domain, which contains an RA domain and a membrane anchoring domain. FrmB plays an important role in cell adhesion, cell migration, and development.

Previous studies have demonstrated that FERM-domain proteins regulate cell-substratum adhesion during cell migration in mammalian cells (Lo, 2006). There are six proteins that contain the FERM domain in *Dictyostelium*: TalinA, TalinB, MyosinVII, FrmA, FrmB, and FrmC (enlazin) (Niewohner et al., 1997; Octtaviani et al., 2006; Tsujioka et al., 1999; Tuxworth et al., 2001).

In *Dictyostelium*, Phg1, Phg2, MyosinVII, and Talin are known as Rap1 downstream effectors, and they all play similar roles in cellular adhesion (Gebbie et al., 2004). TalinB binds to Rap1 via the conserved RA domain (Plak et al., 2016). In this study, I newly generated *frmB* knock-out cells in *Dictyostelium*. FrmB has an RA domain in the FERM domain, which is conserved with other RA domains. The phylogenetic analysis showed that FrmB is closely related to TalinA. These analyses suggest that FrmB has an FERM domain with RA and might function as a Rap1-GTP-binding protein.

Phg2 is required for the cell-substratum interface and cell motility (Gebbie et al., 2004). TalinB is also necessary for adhesion during *Dictyostelium* morphogenesis (Plak et al., 2016). SadA (substrate adhesion-deficient) is the first substratum adhesion receptor and plays an essential role in substratum adhesion during aggregation (Fey et al., 2002; Tarantola et al., 2014). *frmB* null cells show no difference in cell size, but have more filopodia and exhibit weaker adhesion than wild-type cells. These results suggest that FrmB plays an important role in cell-substratum adhesion. *frmB* null cells aggregated normally to form a mound. FrmB is not necessarily required for proper development, but the overexpression of FrmB

inhibits the aggregation of cells during development. This study suggests that FrmB plays some important roles in cell-substratum adhesion, development, and chemotaxis. FrmB contains an RA domain. Further experiments are needed to determine if the RA domain of FrmB binds to Rap1 and other Ras proteins.

IV. CONCLUSION

Cell movement is a coordinated process of F-actin mediated protrusions at the leading edge and myosin-mediated contraction of the rear of a cell. Rap1 is emerging as a major regulator of cytoskeleton reorganization and cell adhesion in *Dictyostelium* chemotaxis. Upon chemoattractant stimulation, Rap1 is rapidly and transiently activated through GPCRs cAR1/cAR3 and G-proteins, predominantly at the leading edge, and contributes to the adhesion of the front of the cell. The activated Rap1 also stimulates phosphorylation and disassembly of myosin II by activation of the Ser/Thr kinase Phg2 at the leading edge of moving cells. Rap1/Phg2-mediated myosin II disassembly at the leading edge of moving cells facilitates F-actin-mediated protrusion of the leading edge, and in part passively allowing myosin II assembly along the lateral sides and posterior of the cell. Rap1 is also involved in stimulation of F-actin polymerization through interconnection with the Rac signaling pathway. The assembled F-actin at the leading edge subsequently recruits Rap1 deactivating proteins such as RapGAP1 to the cell cortex at the front of a cell, leading to deactivation of Rap1 and release of cell adhesion. The deactivated Rap1 is then ready for a new cycle of cytoskeleton reorganization and cell attachment for cell movement (Fig. 1).

In Part I, I have studied the localization of RapGAP3 in migrating cells. RapGAP3 has the I/LWEQ domain, which is known as an F-actin-binding region, in the central region of RapGAP3. It was sufficient for posterior localization in migrating cells, whereas the GAP domain was required for anterior localization. The I/LWEQ domain localized to the cell cortex in an F-actin dependent manner. The I/LWEQ domain of RapGAP3 might bind only to preexisting F-actin at the lateral and posterior regions of the cell but not to newly formed F-actin at the leading edge. This study provides new insights into the posterior localization of signaling molecules in response to chemoattractant stimulation.

In Part II, I have identified and characterized RapGAP6, one of the Rap GAP proteins in *Dictyostelium discoideum*. RapGAP6 appears to play important roles in cell adhesion and morphogenesis, and chemotaxis, but not in development. RapGAP6 was localized in the cytosolic vesicles, suggesting that RapGAP6 might function in the process of phagocytosis.

In Part III, I have examined FrmB, a Rap1 downstream effector containing a RA domain. FrmB has a role in cell-substratum adhesion, development, and chemotaxis.

Overall, Rap1 plays important roles in the dynamic control of cell adhesion by regulating the cytoskeleton during cell migration. Identification and characterization of Rap1-specific GEFs and GAPs would provide new insights into the molecular mechanisms through which Rap1 controls cell adhesion and development during chemotaxis.

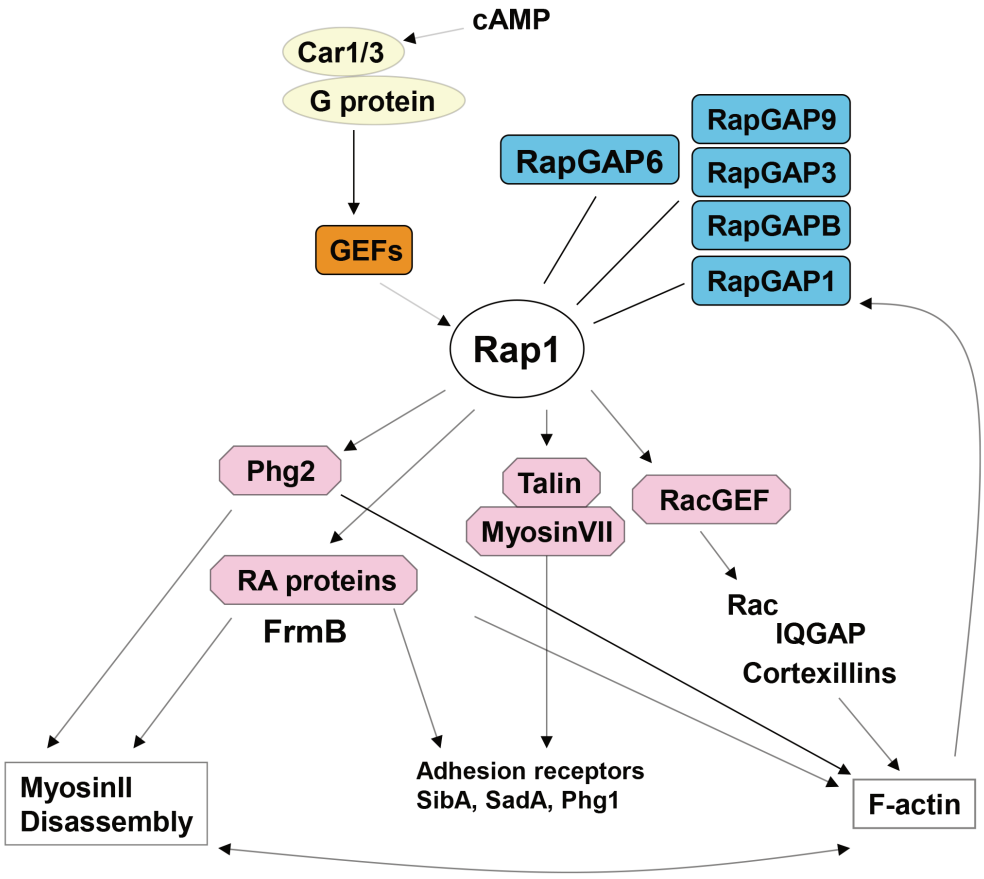

Fig. 1. The Rap1 signaling pathway involved in *Dictyostelium* chemotaxis.

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