The Journal of Microbiology (2012) Vol. 50, No. 4, pp. 555–561
DOI 10.1007/s12275-012-2246-7

REVIEW

Cell Migration: Regulation of Cytoskeleton by Rap1 in *Dictyostelium discoideum*

Mi-Rae Lee and Taeck J. Jeon*

Department of Biology, College of Natural Sciences, Chosun University, Gwangju 501-759, Republic of Korea

(Received May 8, 2012 / Accepted June 29, 2012)

Cell movement involves a coordinated regulation of the cytoskeleton, F-actin-mediated protrusions at the front and myosin-mediated contraction of the posterior of a cell. The small GTPase Rap1 functions as a key regulator in the spatial and temporal control of cytoskeleton reorganization for cell migration. This review outlines the establishment of cell polarity by differential localizations of the cytoskeleton and discusses the spatial and temporal regulation of cytoskeleton reorganization via the Rap1 signaling pathway during chemotaxis with a focus on recent advances in the study of chemotaxis using a simple eukaryotic model organism, *Dictyostelium discoideum*.

Keywords: cell migration, *Dictyostelium*, chemotaxis, Rap1, cytoskeleton

Introduction

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; Ridley *et al.*, 2003; Jin *et al.*, 2009). 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; Williams *et al.*, 2006; Kortholt and van Haastert, 2008). This process is very similar to that of macrophages or neutrophils chasing bacteria. Upon starvation, *Dictyostelium* undergoes a tightly regulated multicellular developmental process in which they secrete cAMP and move toward cAMP via chemotaxis, leading to the eventual formation of fruiting bodies (Chisholm and Firtel, 2004).

The 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).

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 (Sasaki *et al.*, 2004; Kortholt and van Haastert, 2008; Raaijmakers and Bos, 2009). The small GTPase Rap1 has recently been shown to play an important role in regulation of the cytoskeleton during cell migration. This review outlines the asymmetrical distribution of the cytoskeleton during cell migration and summarizes recent advances in the spatial and temporal dynamic regulation of the cytoskeleton by Rap1, primarily focusing on the studies using *Dictyostelium* as a model system.

Asymmetric distribution of the cytoskeleton

Directional cell movement in response to chemoattractant

*For correspondence. E-mail: tjeon@chosun.ac.kr; Tel.: +82-62-230-6654; Fax: +82-62-230-6654

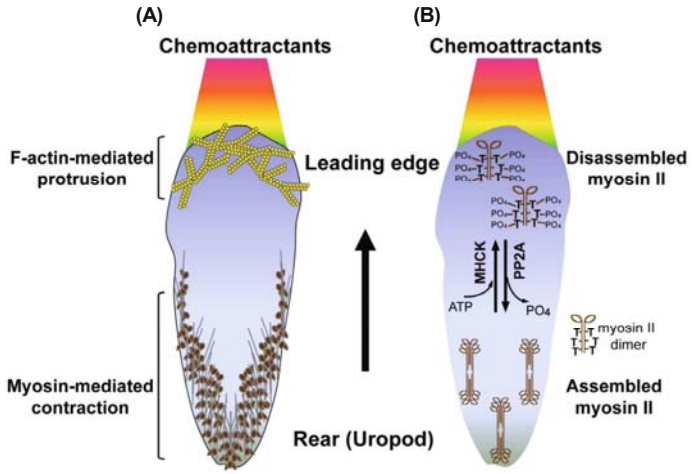

Fig. 1. Asymmetric distribution of the cytoskeleton. (A) Differential localization of cytoskeletons during cell migration. F-actin-mediated protrusion at the leading edge and myosin-ediated contraction at the posterior of a cell. The arrow indicates the direction of cell movement. (B) Regulation of *Dictyostelium* 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).

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).

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 well-known 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 (Ridley *et al*., 2003; Rodal *et al*., 2005; Stephens *et al*., 2008; Firat-Karalar and Welch, 2011). 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 RA-RhoGAP (Yamada *et al*., 2005; Krugmann *et al*., 2006). 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).

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 (De Lozanne and Spudich, 1987; Pasternak *et al*., 1989; Yumura *et al*., 2005; Bosgraaf and van Haastert, 2006). 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, 2007b), and that MHCK-A binds to F-actin and localizes to the leading edge of moving cells to help disassemble the

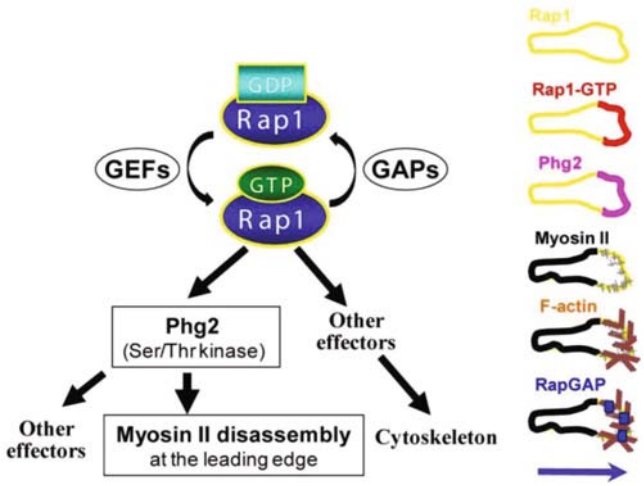

Fig. 2. Myosin II disassembly at the leading edge by Rap1/Phg2 pathway and localization of Rap1 signaling components during chemotaxis. The activation of Rap1 is regulated by GEFs and GAPs. Activated Rap1 stimulates Ser/Thr kinase Phg2, leading to phosphorylation of myosin II and disassembly at the leading 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.

myosin II fibers in this process (Steimle *et al*., 2001).

Rap1 protein

Rap1 is the closest homologue of the small GTPase Ras and cycles between the inactive GDP-bound and active 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 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 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).

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 (Jeon *et al*., 2007b; Cha *et al*., 2010) (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 $PI(4,5)P_2$-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 (Figs. 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*., 2007a, 2007b). 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.

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, 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 (Jeon *et al*., 2007a; Cha *et al*., 2010).

Rap1 GEFs: GbpD has been identified as a Rap1-specific GEF protein. Cells overexpressing GbpD are flat and exhibit strongly increased cell-substrate 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 (Goldberg *et al*., 2002; Bosgraaf *et al*., 2005; 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

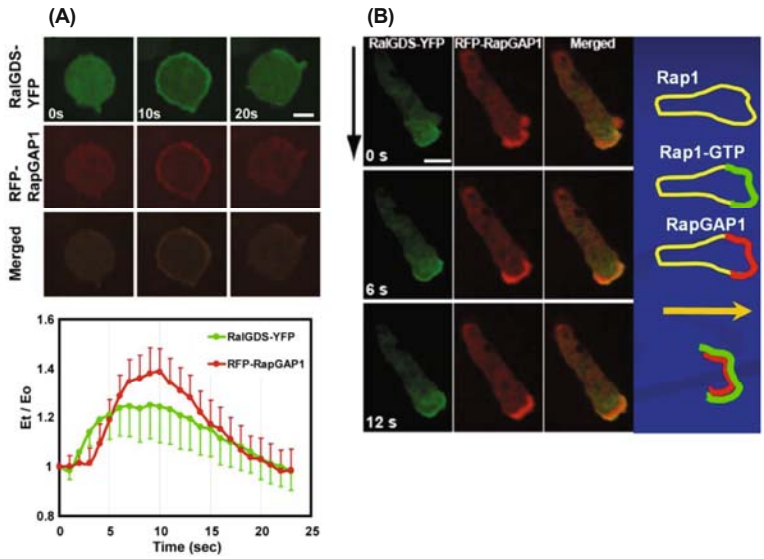

Fig. 3. Localization of Rap1-GTP and RapGAP1. (A) Translocation of the two proteins to the cell cortex upon chemoattractant stimulation. Activated Rap1 and RapGAP1 were labeled by RalGDS-YFP and RFP, respectively. The intensity of the fluorescence of the protein at the cell cortex was quantified and 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)].

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 RalGDS-YFP 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 (Lee *et al*., 2010; Cha and Jeon, 2011).

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).

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 contribute 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. 4). 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 and further insight into the molecular mechanisms through which Rap1 controls cell adhesion during chemotaxis should be accomplished in the near future.

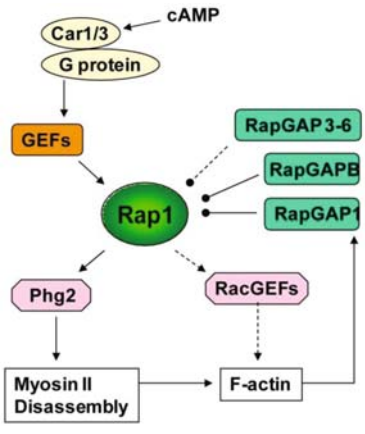

Fig. 4. The Rap1 signaling pathway involved in *Dictyostelium* chemotaxis.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0065992 and 2011-0022220).

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