Regulation of Actin Cytoskeleton by Rap1 Binding to RacGEF1

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Rap1 is rapidly and transiently activated in response to chemoattractant stimulation and helps establish cell polarity by locally modulating cytoskeletons. Here, we investigated the mechanisms by which Rap1 controls actin cytoskeletal reorganization in *Dictyostelium* and found that Rap1 interacts with RacGEF1 *in vitro* and stimulates F-actin polymerization at the sites where Rap1 is activated upon chemoattractant stimulation. Live cell imaging using GFP-coronin, a reporter for F-actin, demonstrates that cells expressing constitutively active Rap1 (Rap1CA) exhibit a high level of F-actin uniformly distributed at the cortex including the posterior and lateral sides of the chemotaxing cell. Examination of the localization of a PH-domain containing PIP3 reporter, PhdA-GFP, and the activation of Akt/Pkb and other Ras proteins in Rap1CA cells reveals that activated Rap1 has no effect on the production of PIP3 or the activation of Akt/Pkb and Ras proteins in response to chemoattractant stimulation. Rac family proteins are crucial regulators in actin cytoskeletal reorganization. *In vitro* binding assay using truncated RacGEF1 proteins shows that Rap1 interacts with the DH domain of RacGEF1. Taken together, these results suggest that Rap1-mediated F-actin polymerization probably occurs through the Rac signaling pathway by directly binding to RacGEF1.

INTRODUCTION

The differential polymerization of F-actin allows for protrusions at the front of the cell, followed by a posterior retraction via myosin II assembly (Bagorda and Parent, 2008; Ridley et al., 2003). Actin polymerization is mediated by the Arp2/3 complex, the activity of which is controlled by adaptor proteins of the WASP and SCAR/WAVE families. Rac proteins belong to Rho family small GTPases and bind to the SCAR/WAVE complexes, stimulating F-actin assembly during pseudopod extension (Ibarra et al., 2005; Ridley et al., 2003; Sasaki and Firtel, 2006). RacB in *Dictyostelium* is activated upon chemoattractant stimulation and involved in chemoattractant-mediated F-actin polymerization. RacB activation is partly controlled by the PI3K pathway. RacGEF1 has specificity for RacB and localizes to sites of

F-actin polymerization (Park et al., 2004; Stephens et al., 2008).

Phosphoinositide 3-kinases (PI3Ks) have been demonstrated to play important roles in the directional sensing and the establishment of cell polarity in chemotaxis, even though recent studies suggest the importance of several interconnecting pathways to mediate chemotaxis (Chen et al., 2007; Kolsch et al., 2008; van Haastert et al., 2007). The reciprocal localization and activation of PI3K and PTEN leads to the accumulation of phosphatidylinositol (3,4,5) trisphosphate (PIP3) at the leading edge that function as docking sites for diverse pleckstrin homology (PH) domain-containing proteins, including Akt/Pkb, CRAC, and PhdA. These recruited PH-domain containing proteins contribute to establishing cell polarity and F-actin polymerization during chemotaxis. PI3K contains a Ras binding domain (RBD), which interacts with Ras proteins (Funamoto et al., 2002; Sasaki et al., 2004).

Rap1 is a small GTPase and 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 (Bos, 2005; Kooistra et al., 2007; Raaijmakers and Bos, 2009). In *Dictyostelium*, Rap1 is rapidly and transiently activated in response to chemoattractant stimulation at the leading edge of chemotaxing cells and helps establish cell polarity by locally modulating myosin II assembly and disassembly through the Rap1/Phg2 signaling pathway (Cha et al., 2010; Jeon et al., 2007a). Spatial and temporal regulation of Rap1 activity by Rap1 GAPs is required for proper cell migration as well as cell differentiation and development (Jeon et al., 2007b; 2009; Parkinson et al., 2009). Rap1 has also been linked to the control of F-actin polymerization. In mammalian cells, Rap1 promotes cell spreading by binding to and localizing the RacGEFs Vav2 and Tiam1 to the sites of active lamellipodia extension (Arthur et al., 2004). In *Dictyostelium*, F-actin assembly was increased in the cells expressing constitutively active Rap1 (Jeon et al., 2007a; 2007b). However, the mechanism by which Rap1 controls actin cytoskeletal reorganization in *Dictyostelium* has not been addressed.

To understand the mechanism for the regulation of actin cytoskeletal reorganization by Rap1 in *Dictyostelium*, we examined the effects of Rap1 activation on the PI3K signaling pathway and the interaction of Rap1 with Rac signaling components. Our data suggest that activated Rap1 mediates F-actin polym-

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erization by directly binding to a RacGEF protein.

MATERIALS AND METHODS

Materials

Glutathione-sepharose beads were purchased from Amersham Biosciences, H2B from Boehringer Mannheim, [γ - 32 P] ATP from ICN Biomedicals, and Protein A-Sepharose CL-4B from Pharmacia Biotech., Inc. A monoclonal anti-Myc antibody and a monoclonal anti-pan-Ras (Ab-3) antibody were obtained from Santa Cruz Biotechnology and Oncogene Research Products, respectively. An anti-PKB antibody was described previously (Meili et al., 1999).

Cell culture, strains, and plasmids

Dictyostelium cells, strain KAX-3, were cultured axenically in HL5 medium at 22°C. The expression plasmids for GFP-PhdA, GFP-coronin, RalGDS-YFP, and RFP-coronin were described previously (Funamoto et al., 2001; Jeon et al., 2007b). The plasmids were transformed by the electroporation method. Transformants were maintained in 20 μ g/ml G418, 50 μ g/ml hygromycin, or both as required.

Biochemical assays

Akt/Pkb activity assay: Akt/Pkb activity was measured in an immunocomplex kinase assay following immunoprecipitation with an anti-PKB antibody as described previously (Meili et al., 1999). Endogenous Akt was immunoprecipitated and subjected to an *in vitro* kinase assay using H2B as a substrate.

Ras activation assay: The Ras binding domain (RBD) of mammalian Raf1 was expressed in *Escherichia coli* as a GST fusion protein and purified using glutathione-sepharose beads as described previously (Franke et al., 1997). The active Ras proteins were pulled down by the purified GST-RBD of Raf1 conjugated with glutathione-sepharose beads and then detected in an immunoblot assay using an anti-pan-Ras antibody.

Pull-down binding assay: We amplified the DNA sequence of the DH or PH domains in RacGEF1 and cloned them into the pGEX6P-1 expression vector. The expression vector containing the DNA sequence encoding amino acids 763-983 was named pGST-DH, which includes only the DH domain. pGST-DH/PH or pGST-DH/PHE plasmids contain the DNA sequences encoding amino acids 763-1097 or 763-1219, respectively, which include the DH domain and PH domains of RacGEF1. The GST-tagged fusion proteins were purified using glutathione-coupled sepharose beads and used in the pull-down assay. The cells expressing Myc-tagged wild-type Rap1 (OE) or constitutively active Rap1 (CA) were lysed and then incubated for 20 min with the GST-fusion truncated RacGEF1 or GST protein without any fusion protein as a control. The mixture was washed three times to remove proteins unbound to GST-fusion proteins, subjected to SDS-PAGE, and then visualized by Western blotting using a monoclonal antibody against the Myc tag.

Image acquisition

The subcellular localization of proteins in response to chemoattractant stimulation was examined as previously described (Jeon et al., 2007b; 2009). The 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 nM cAMP at 6-min intervals for 5 h. The pulsed cells were plated on glass-bottomed microwell plates. For imaging chemotaxing cells, a micropipette filled with 150 μ M cAMP was positioned near the cells. For analysis of translocation kinetics of

the protein to the cell cortex in response to cAMP stimulation, the pulsed cells were exposed to uniform chemoattractant stimulation and then fluorescence images were taken at time-lapse intervals of 1 s for 1 min using a confocal microscope (DMIRE2; Leica) with HCX Plan Apo NA 1.40 100 $\times$ or 63 $\times$ objective lenses (oil CS; Leica) and a camera (EM-CCD or ORCA-ER; Hamamatsu Photonics). Images were captured using SimplePCI software (Compix Inc., Imaging Systems) and were analyzed using ImageJ software (NIH).

RESULTS

F-actin polymerization is induced at the sites where Rap1 is activated in response to chemoattractant stimulation.

The cells expressing constitutively active Rap1 (Rap1CA cells) or *rapGAP1* null cells exhibit high levels of F-actin, compared to wild-type cells, even though the chemoattractant-mediated F-actin polymerization kinetics are similar between Rap1CA cells or *rapGAP1* null cells and wild-type cells (Jeon et al., 2007b; 2007b). To understand the mechanism by which Rap1 regulates F-actin polymerization, we investigated the *in vivo* chemoattractant-mediated F-actin polymerization by monitoring the subcellular localization of coronin, a marker protein for F-actin assembly (Gerisch et al., 1995; Jeon et al., 2007b; Rodal et al., 2005), in Rap1CA cells and wild-type cells.

Unstimulated wild-type cells displayed low levels of coronin-GFP at the cortex. Upon uniform chemoattractant stimulation, cytosolic coronin-GFP transiently translocated to the cell cortex with a peak at ~ 10 s (Fig. 1A), followed by delocalization within ~ 30 s, as previously reported (Jeon et al., 2007b), suggesting that F-actin assembly at the cell cortex is transiently induced by chemoattractant stimulation. In contrast, Rap1CA cells exhibited a higher basal level of coronin-GFP at the cell cortex and had response kinetics similar to those of wild-type cells, with a slightly lower maximum level of cortical coronin caused by the higher basal level, indicating that Rap1CA cells have increased levels of F-actin even before stimulation. In chemotaxing wild-type cells, coronin-GFP localized to the leading edge, whereas the protein was found uniformly all around the cell cortex, including the posterior and lateral sides of the cells, in chemotaxing Rap1CA cells (Fig. 1B). The contemporaneous examination of both the Rap1-GTP reporter RalGDS-YFP and a marker of F-actin RFP-coronin in a chemotaxing cell showed the colocalization of the two proteins at the leading edge, even though there was a small amount of RFP-coronin at the posterior (Fig. 1C). In consistent with the previous results (Jeon et al., 2007a), these results suggest that F-actin polymerization is induced at the sites of Rap1 activation.

The activation kinetics of PI3K and Akt/Pkb upon chemoattractant stimulation in Rap1CA cells

The PI3K signaling pathway is linked to F-actin polymerization. To determine whether Rap1 regulates F-actin polymerization through the PI3K signaling pathway, we examined the activities of PI3Ks and Akt/Pkb in Rap1CA cells. Upon chemoattractant stimulation, PI3Ks are rapidly activated and produce PIP3 at the membrane that acts as binding sites for the PH domain-containing proteins (Funamoto et al., 2002; Kolsch et al., 2008; Ridley et al., 2003). The activities of PI3Ks can be visualized by monitoring the localization of PH domain-containing proteins. We first compared the translocation kinetics of PhdA-GFP, a PH domain containing PIP3 reporter (Funamoto et al., 2001; 2002), to the cell cortex upon chemoattractant stimulation in wild-type cells and Rap1CA cells. There was no apparent dif-

Fig. 1. Localization of coronin, a marker of F-actin. (A) Translocation of GFP-coronin in wild-type KAx-3 cells and cells expressing constitutively active Rap1 (Rap1CA) to the cell cortex in response to uniform chemoattractant stimulation. Fluorescent images of GFP-coronin at the indicated times were obtained from time-lapse recordings and quantified as previously described (Jeon et al., 2007b). The graphs represent the mean of data from several cells at videos taken during at least three separate experiments. Error bars represent SD. (B) Spatial localization of GFP-coronin in chemotaxing wild-type and Rap1CA cells. (C) Colocalization of RalGDS-YFP, a Rap1-GTP reporter, and RFP-coronin, a marker protein of newly formed F-actin, in chemotaxing cells.

Fig. 2. Localization of PhdA-GFP and Akt/Pkb activation. (A) Translocation of PhdA-GFP to the cell cortex upon uniform chemoattractant stimulation in wild-type cells and Rap1CA cells. Fluorescent images of PhdA-GFP were obtained from time-lapse recordings and quantified and graphed as described in Fig. 1. (B) Akt/Pkb activation. Aggregation-competent cells were treated with 10 μ M cAMP for the indicated times. Akt/Pkb proteins were immunoprecipitated with anti-Akt antibodies and the activity of Akt/Pkb was assayed using histone2B (H2B) as a substrate (see "Materials and Methods"). Akt protein levels were determined in each sample by Western blot analysis and shown at the bottom panel.

ference in the translocation kinetics of PhdA between wild-type cells and Rap1CA cells (Fig. 2A). Upon chemoattractant stimulation, PhdA was rapidly and transiently translocated to the cell cortex with a peak at around 7 s and then it delocalized within ~20 s in both wild-type and Rap1CA cells. This indicates that the kinetics of PIP3 production in response to chemoattractant stimulation and the PI3K activity in Rap1CA cells are similar to those in wild-type cells.

Next we measured the activation levels of Akt/Pkb, which is another downstream effector of PI3K (Meili et al., 1999; Sasaki and Firtel, 2006), upon chemoattractant stimulation in wild-type cells and Rap1CA cells. The two strains showed similar activation kinetics of Akt/Pkb in response to cAMP stimulation. The rapid and transient activation of Akt/Pkb upon chemoattractant

stimulation was found in both cell lines and the elevated level decreased to the basal level within 40 s, even though Rap1CA cells showed a little lower maximal level of Akt/Pkb activity compared to wild-type cells (Fig. 2B). These results indicate that activated Rap1 has no effect on the production of PIP3 or the activation of Akt/Pkb in response to chemoattractant stimulation, suggesting that the regulation of F-actin polymerization by Rap1 is not through the PI3K signaling pathway, at least PIP3 and Akt/Pkb.

Chemoattractant-mediated Ras activation kinetics in Rap1CA cells

Several studies have shown that Ras proteins are essential for cAMP-mediated chemotaxis in *Dictyostelium*. Cells disrupted in

Fig. 3. Ras activation upon uniform chemoattractant stimulation. Ras activation in wild-type and Rap1CA cells. Cells were stimulated with cAMP for the indicated time points and then lysed, and the activated Ras proteins were pulled down with GST-RBD of Raf1 and detected by immunoblot analysis using an anti-pan-Ras antibody. As a control for the input amount of total Ras proteins, some amount of the cells right after lysis was taken and analyzed at the bottom panel.

both *rasC* and *rasG* have no cAMP relay and fail to chemotax towards cAMP (Bolourani et al., 2006; 2008). To determine if Rap1 indirectly regulates F-actin polymerization through Ras proteins, we examined the activation of Ras proteins, by using an anti-pan-Ras antibody that recognizes some or all *Dictyostelium* Ras proteins, in response to chemoattractant stimulation in Rap1CA cells. Ras proteins were rapidly and transiently activated in response to cAMP stimulation in both wild-type cells and Rap1CA cells with similar activation kinetics (Fig. 3). This indicates that Ras activation was not affected by activated Rap1 and that Ras proteins are unlikely to be involved in the regulation of F-actin polymerization by Rap1. However, as found in the experiments measuring Akt/Pkb activation levels, Rap1CA cells exhibited slightly lower activation levels of Ras proteins than wild-type cells.

Rap1 interacts with RacGEF1 *in vitro*

Members of the Rac small GTPases are key regulators of cytoskeletal dynamics. Activated Rac proteins stimulate downstream effectors such as SCAR/WAVE and WASP proteins that mediate F-actin polymerization (Ridley et al., 2003; Sasaki and Firtel, 2006). It was recently demonstrated that Rap1 interacts with RacGEFs such as Vav2 and Tiam1 in mammalian cells (Arthur et al., 2004; Raaijmakers and Bos, 2009). In *Dictyostelium*, RacB has been shown to play an important role in the regulation of F-actin polymerization and RacGEF1 was identified as a RacB specific GEF protein (Park et al., 2004). RacGEF1 localizes to the sites of F-actin assembly (Park et al., 2004). To test the possibility that Rap1 mediates F-actin polymerization through the Rac signaling components, we assayed the interaction of Rap1 with RacGEF1 *in vitro*. RacGEF1 has an N-terminal CH domain and a C-terminal DH and PH domain (Fig. 4A). To examine the interaction between RacGEF1 and Rap1, we prepared several truncated RacGEF1 proteins containing the DH domain (Fig. 4A) by expressing in *E. coli* as GST fusion proteins. The purified GST-fusion truncated RacGEF1 proteins were used in the pull down assay. The lysates of cells expressing myc-tagged Rap1 or Rap1CA were mixed with the purified GST-fusion recombinant proteins, followed by purification of the GST-fusion proteins using sepharose-conjugated beads. The purified Rap1 was detected using anti-myc antibodies. When GST alone was used in the assay, there was no Rap1 detected (Fig. 4B). In contrast, the GST-DH, GST-DH/PH, or GST-DH/PHE proteins were able to pull down both myc-Rap1 and myc-Rap1CA proteins. The lysates from myc-Rap1CA expressing cells exhibited a stronger Rap1 band.

Fig. 4. Interaction of Rap1 with RacGEF1 *in vitro*. (A) Schematic diagram of truncated RacGEF1 proteins. Domain structures of RacGEF1 and truncated RacGEF1 proteins are shown. (B) Pull-down assay using GST-fusion truncated RacGEF1. The GST-fusion truncated proteins bound to glutathione-sepharose beads were incubated with lysates from cells expressing myc-tagged wild-type Rap1 (OE) or constitutively active Rap1 (CA). After the beads were washed to remove the unbound material, the samples were subjected to SDS-PAGE and immunoblotted with an anti-myc antibody shown in the upper panel. GST-RBD of RaiGDS and GST alone were used as positive and negative controls, respectively. The membrane used in the immunoblot assay was stained with Coomassie blue to control for the input amount of GST-fusion proteins shown in the lower panel. (C) Some of the cells before the pull-down assay were immunoblotted as a control of the input amount of myc-tagged Rap1 proteins.

These results suggest that Rap1 interacts with RacGEF1 through the DH domain *in vitro* and that activated Rap1 has a higher binding affinity to the DH domain.

DISCUSSION

Our study demonstrates that Rap1 binds to the DH domain of RacGEF1, which has GEF specificity for RacB, in the *in vitro* binding assay and suggests that activated Rap1 mediates F-actin polymerization through the Rac signaling pathway independently of the PI3K signaling. Our *in vivo* localization assay using GFP-coronin, a reporter protein for a newly formed F-actin, allowed us to examine the dynamic changes of F-actin assembly in response to chemoattractant stimulation and the effects of activated Rap1 on the responses in live cells. The analysis of the kinetics of F-actin assembly using GFP-coronin showed that there is a higher level of F-actin at the cell cortex in cells expressing constitutively active form of Rap1 (Rap1CA cells) than wild-type cells. Chemotaxing Rap1CA cells exhibited newly formed F-actin all around the cell cortex, whereas chemotaxing wild-type cells displayed one major F-actin assembly at the leading edge. RFP-coronin colocalized with the activated Rap1 reporter RaiGDS-YFP at the leading edge of a chemotax-

Fig. 5. Model for F-actin polymerization mediated by Rap1 signaling. Upon chemoattractant stimulation, Rap1 is rapidly and transiently activated. The activated Rap1 (Rap1-GTP) facilitates myosin II disassembly through Phg2, a Ser/Thr kinase, allowing F-actin polymerization at the leading edge of chemotaxing cells (Jeon et al., 2007a). In addition, the activated Rap1 is also likely involved in stimulation F-actin polymerization through the Rac signaling pathway. Direct interaction of Rap1-GTP with RacGEF1 might contribute to the polymerization of F-actin by RacGEF1/RacB signaling (Park et al., 2004). The unbroken lines represent the pathways demonstrated previously (Jeon et al., 2007a; 2007b; Park et al., 2004), and the dotted lines should be further confirmed.

ing cell. These results indicate that activated Rap1 induces F-actin polymerization at the sites of Rap1 activation, which is consistent with those previously reported based on the biochemical F-actin polymerization assay and chemotaxis analysis (Jeon et al., 2007a; 2007b). It has been reported previously that Rap1CA cells or *rapGAP1* null cells contain higher levels of F-actin than do wild-type cells and have an increased production of lateral pseudopodia, protrusions of plasma membrane by F-actin assembly, and a decreased formation of a prominent leading edge in the direction of the chemoattractant gradient (Jeon et al., 2007a; 2007b).

PI3Ks are important for regulating F-actin assembly during chemotaxis and are necessary for the membrane localization of the PH-domain containing proteins such as Akt/Pkb and PhdA (Funamoto et al., 2001; 2002; Kolsch et al., 2008; Sasaki and Firtel, 2006). PhdA is required for proper F-actin organization (Funamoto et al., 2001). It has been reported that *Dictyostelium pi3k1/2* null cells or wild-type cells treated with LY294002, an inhibitor for PI3Ks, exhibit a 30% decrease in chemoattractant-mediated F-actin assembly (Funamoto et al., 2001; 2002). PI3Ks have Ras binding domains and the PI3K activity is regulated by Ras proteins (Funamoto et al., 2002; Sasaki et al., 2004). Therefore, it was postulated that Rap1 mediates F-actin polymerization through the PI3K pathway. However, our data demonstrate that Rap1 has no effect on the production of PIP3, which is indicated by the localization of PhdA-GFP as well as Akt/Pkb activity in response to chemoattractant stimulation. The translocation kinetics of PhdA-GFP to the cell cortex in response to chemoattractant stimulation or the Akt/Pkb activity in Rap1CA cells is almost indistinguishable to those in wild-type cells. We suggest that Rap1 mediates F-actin assembly in a

PI3K-independent pathway. However, to exclude a possibility that other effectors of PI3K might be affected by Rap1, we would need to test directly for PI3K activity in Rap1CA cells or *rap1* null cells.

It has been reported in *Dictyostelium* that two, RasC and RasG, of six Ras subfamily members identified so far are activated in response to cAMP stimulation and involved in cAMP-mediated chemotaxis and actin cytoskeleton reorganization (Kae et al., 2004; Kortholt and van Haastert, 2008). The Ras activation assay indicates that chemoattractant-mediated Ras activation kinetics are not affected by activated Rap1, since Rap1CA cells exhibit similar Ras activation kinetics in response to chemoattractant stimulation and a slightly lower maximal level of activated Ras proteins, compared to wild-type cells. Therefore, it appears that Rap1 induces F-actin polymerization independently of other Ras proteins such as RasC and G, which are known as regulators of chemoattractant-mediated F-actin assembly. These results are in agreement with our previous results that PI3K pathway is not affected by activated Rap1.

Several studies have reported that Rap proteins are directly linked to Rho family proteins through the interaction with the RacGEFs such as Vav2 and Tiam1 and the RhoGAP Arap3. In mammalian cells, Rap1 interacts with the DH-PH domain of the RacGEFs, Vav2 and Tiam1 and an RA domain of Arap3 (Arthur et al., 2004; Gerard et al., 2007; Krugmann et al., 2006). Here, we demonstrate that *Dictyostelium* Rap1 interacts with the DH domain of RacGEF1 even though the importance of the DH domain in the interaction with Rap1 should be further examined, and that activated Rap1 shows a higher binding affinity to RacGEF1. Since the activation levels of Rap1 increase upon chemoattractant stimulation, we expect that the interaction between Rap1 and RacGEF1 would also increase in response to the stimulation. The subcellular localization of RacGEF1 was examined by Park et al. (2004). Upon uniform chemoattractant stimulation, cytosolic GFP-RacGEF1 transiently translocates to the cell cortex, and, in chemotaxing cells, RacGEF1 preferentially localizes to the leading edge, similar to F-actin assembly (Park et al., 2004). We examined that GFP-RacGEF1 and RFP-coronin, a marker for F-actin, colocalize at the sites of F-actin foci (Uchida and Yumura, 2004) at the bottom of the cell and that both proteins exhibit similar translocation kinetics to the cell cortex in response to chemoattractant stimulation (Unpublished data). *racGEF1* null cells exhibit reduced RacB activation, suggesting that RacGEF1 has specificity for RacB (Park et al., 2004). RacB is a regulator for chemoattractant-mediated F-actin polymerization. Even though the exact roles of the interaction of Rap1 with RacGEF1 should be further examined, based on previously reported findings and our present results, we suggest that Rap1 mediates F-actin polymerization possibly through RacGEF1 and RacB.

In conclusion, upon chemoattractant stimulation, Rap1 is rapidly activated and controls actin cytoskeleton organization in two ways (Fig. 5). First, the activated Rap1 leads to the disassembly of myosin II by binding and activating Phg2 and passively allowing F-actin polymerization at the leading edge of chemotaxing cells (Jeon et al., 2007a; 2007b). In the second way that is suggested by our present study, Rap1 is likely to be involved in F-actin polymerization by directly binding RacGEF1 and possibly activating RacB, which should be further examined. F-actin assembly also might be controlled by several unknown Rap1 effectors. Additional experiments are in progress to determine if Rap1 binds to intact RacGEF1 *in vivo* and the mechanisms by which Rap1 modulates RacGEF1 or RacB activities.

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