Introduction

Cell migration is an integrated process that requires the continuous, coordinated formation and disassembly of cell attachments (Ridley et al., 2003). The migratory cycle includes extension of an F-actin–mediated protrusion, formation of stable attachments near the leading edge, translocation of the cell body forward, release of the attachments, and retraction of the cell’s posterior. Integrin-mediated cell adhesion is an important adhesion mechanism in mammalian cells and plays key roles in cell spreading and migration (Schwartz, 2001). In Dictyostelium discoideum, several cell-surface proteins have been linked to cell adhesion (Fey et al., 2002; Chisholm and Firtel, 2004; Cornillon et al., 2006).

The small GTPase Rap1 regulates integrin-mediated cell adhesion and may control both integrin activity (affinity) and integrin clustering (avidity; Kinbara et al., 2003; Bos, 2005). In D. discoideum, Rap1 has been linked to cytokinematic regulation of cell movement, phagocytosis, and the response to osmotic stress (Rebstein et al., 1997; Kang et al., 2002; Jeon et al., 2007). Rap1 is rapidly and transiently activated in response to chemoattractant stimulation, and the activated Rap1 localizes at the leading edge of chemotaxing cells (Jeon et al., 2007). Rap1 regulates cell adhesion and helps establish cell polarity by locally modulating MyoII assembly and disassembly through Phg2, a Rap1-GTP–mediated Ser/Thr kinase, which may control myosin heavy chain kinases (Steimle et al., 2001; Jeon et al., 2007). The mechanisms underlying the spatial and temporal regulation of Rap1 activation and deactivation are not yet understood.

Here, we demonstrate that a specific D. discoideum Rap GAP (GAP; RapGAP1) that helps mediate cell adhesion by negatively regulating Rap1 at the leading edge. Defects in spatial regulation of the cell attachment at the leading edge in rapGAP1− (null) cells or cells over-expressing RapGAP1 (RapGAP1OE) lead to defective chemotaxis. rapGAP1− cells have extended chemoattractant-mediated Rap1 activation kinetics and decreased MyoII assembly, whereas RapGAP1OE cells show reciprocal phenotypes. We see that RapGAP1 translocates to the cell cortex in response to chemoattractant stimulation and localizes to the leading edge of chemotaxing cells via an F-actin–dependent pathway. RapGAP1 localization is negatively regulated by Ctx, an F-actin bundling protein that functions during cytokinesis. Loss of Ctx leads to constitutive and uniform RapGAP1 cortical localization. We suggest that RapGAP1 functions in the spatial and temporal regulation of attachment sites through MyoII assembly via regulation of Rap1–guanosine triphosphate.

Results and discussion

RapGAP1 exhibits Rap1-specific GAP activity

Because rap1 is an essential gene in D. discoideum, genetic modulation of Rap1’s activity through regulators, such as GAPs, would provide a mechanism for examining Rap1 function. RapGAP1 (DictyBaseID: DDB0233726) is one of nine ORFs in the D. discoideum genome with a Rap1 GAP domain (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1). RapGAP1 has a C-terminal RapGAP domain and an N-terminal

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Abbreviations used in this paper: GAP, GTPase–activating protein; GDS, guanine nucleotide dissociation inhibitor; PI3K, phosphoinositide-3 kinase; RBD, Rap binding domain.

The online version of this paper contains supplemental material.
Figure 1. RapGAP1 negatively regulates cell adhesion and cell spreading. (A) Rap1-GTP stability assay using the GST fusion GAP domain of RapGAP1. (B) Rap1 activation kinetics in response to chemoattractant stimulation. (C) Extended time points from B (Materials and methods). (D) Spreading morphology of vegetative cells. Bar, 10 μm. (E) Cell-substratum adhesion assays. Adhesion was measured by the ratio of detached cells to the total number of cells. (F) Kinetics of F-actin polymerization and MyoII assembly in the Triton X-100-insoluble cytoskeletal fraction of the cells in response to chemoattractant stimulation. (G) Analysis of chemotaxis using DIAS software. Representative stacked images are shown. Superimposed images were taken every 1 min. The arrow indicates the direction of movement. Data represents mean ± SD from at least three experiments.
domain predicted to be homologous in structure to the F41 fragment of flagellin (Samatey et al., 2001). RapGAP1 is expressed at all stages of development (Fig. S1 B).

To examine whether RapGAP1 exhibits GAP activity for Rap1, we assayed the recombinant GAP domain’s ability to stimulate the GTPase activity of Rap1 and Ras proteins (Fig. 1 A). When purified GST-GAP domain was added to cell lysates, there was a decreased level of active Rap1, which was dependent on the amount of GST-GAP added, whereas the level of active Ras was unaffected. GST alone caused a minimal change in Rap1-GTP levels. These data indicate that the GAP domain of RapGAP1 possesses GAP activity for Rap1 but not for the Ras proteins examined.

**RapGAP1 affects the kinetics and level of Rap1 activation**

A rapGAP1-null (rapGAP1−) strain exhibited no defect in growth or multicellular development (Materials and methods; unpublished data). To determine whether RapGAP1 regulates Rap1-GTP in vivo, we studied the chemoattractant-stimulated kinetics of Rap1 activation in wild-type, rapGAP1−, and rapGAP1OE cells overexpressing GFP-RapGAP1 (RapGAP1OE; Fig. 1, B and C). We observed striking differences in GFP-RapGAP1OE cells. Even though the Rap1 activation kinetics are similar to those of wild-type cells, the amplitude of Rap1 activation and Rap1-GTP levels in unstimulated RapGAP1OE cells are reduced. In contrast, Ras activation is unaffected (Fig. S1 C), indicating that RapGAP1 has Rap1-specific GAP activity in vivo and regulates Rap1-GTP levels. Thus, overexpression of RapGAP1 provides the first functional approach to negatively regulate Rap1-GTP levels in *D. discoideum* and understand the phenotypes that result from reduced Rap1 activity.

**RapGAP1 regulates cell attachment, cell spreading, and MyoII assembly through Rap1**

Cells expressing constitutively active Rap1 (Rap1G12V) are flat, spread, and show strong attachment to the substratum (Jeon et al., 2007). rapGAP1− cells have similar phenotypes; they are more flattened and spread than wild-type cells and display an ~50% increase in cell attachment (Fig. 1, D and E). These phenotypes are complemented in GFP-RapGAP1OE cells, suggesting that RapGAP1 is involved in reorganizing the cell cytoskeleton and cell–substratum attachment. As vegetative cells were used in these assays, we examined basal Rap1-GTP levels in attached vegetative cells (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1). rapGAP1− cells exhibited a moderate increase (~30%) in the level of Rap1-GTP, whereas we detected no Rap1-GTP in our assay of GFP-RapGAP1OE cells, presumably because of Rap1-GTP hydrolysis by elevated RapGAP1. Our findings suggest that RapGAP1 negatively regulates cell adhesion by deactivating Rap1. GFP-RapGAP1OE cells, which exhibit no detectable activated Rap1 in our assay, are still attached, suggesting there is at least one additional mechanism of cell attachment that is not regulated by Rap1. A major function of Rap1 in resting cells may be to control cell spreading; the increase in attachment observed in rapGAP1− or Rap1G12V cells may result from increased surface area and a parallel increase in attachment sites. It is noteworthy that the phenotypes of rapGAP1− cells are moderate compared with those of Rap1G12V cells. We suggest that the differences might result from the differences in the level and timing of Rap1 activation. rapGAP1− cells show extended kinetics of chemoattractant-mediated Rap1 activation (Fig. 1, B and C), whereas Rap1G12V cells exhibit a high level of GTP-bound Rap1G12V in unstimulated cells, with no change in that level upon stimulation. As cells expressing Rap1G12V exhibit severely delayed chemoattractant-mediated MyoII assembly (Jeon et al., 2007), we investigated the effect of RapGAP1 on chemoattractant-mediated reorganization of the cytoskeleton (Fig. 1 F). In wild-type cells, chemoattractant stimulation results in a small decrease in assembled MyoII in the Triton X-100–insoluble fraction, followed by a transient increase in cortical MyoII with a peak at ~30 s (Steimle et al., 2001; Jeon et al., 2007). We find that this increase in cortical MyoII is reduced in rapGAP1− cells, consistent with our findings in Rap1G12V cells (Jeon et al., 2007). In contrast, RapGAP1OE cells exhibit a higher basal level of assembled MyoII and have response kinetics similar to those of wild-type cells, with the higher maximum level of cortical MyoII caused by the higher basal level. We suggest that RapGAP1 regulates MyoII assembly at the cell cortex through negative regulation of Rap1-GTP levels.

Wild-type cells exhibit a biphasic F-actin polymerization profile with a sharp peak at 5 s and a second lower, broader peak linked to pseudopod extension at ~45–60 s (Fig. 1 F; Hall et al., 1989). RapGAP1OE and wild-type cells display similar kinetics of F-actin polymerization. rapGAP1− cells have a slightly elevated basal level of F-actin and a similar first peak. However, F-actin levels do not subsequently decrease to the basal level, as in wild-type cells, and they exhibit a proportionally elevated second peak (Fig. 1 F). Thus, a reduction of the chemoattractant-mediated MyoII assembly permits an enhanced F-actin response, consistent with a model in which assembled MyoII is antagonistic to F-actin polymerization and leading edge functions. GFP-RapGAP1 complements the altered F-actin assembly in rapGAP1− cells.

**RapGAP1 controls cell adhesion at the front of chemotaxing cells**

We analyzed the ability of rapGAP1 mutant strains to polarize and chemotax up a chemoattractant gradient using 2D DIAS software (Wessels et al., 1998). Fig. 1 G and Videos 1 and 2 (available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1) show that rapGAP1− cells have a small loss of polarity (increased roundness) and move slightly slower than wild-type cells. This is similar to, but not as severe as, the effect of overexpressing Rap1G12V (Jeon et al., 2007).
Figure 2. Localization of RapGAP1. (A) Translocation of GFP-RapGAP1 in wild-type cells to the cell cortex in response to uniform chemoattractant stimulation and localization of GFP-RapGAP1 during chemotaxis (bottom). The arrow indicates the direction of movement. (B–D) Dual-view analyses of the cells expressing bothRalGDS-YFP and RFP-RapGAP1. (B) Translocations of the two proteins to the cell cortex by chemoattractant stimulation were contemporaneously imaged using a dual-view splitter. The merged image at 10 s after cAMP stimulation was enlarged in the bottom picture and the fluorescence intensities were measured along a line through the central portion of the cell. (C) Translocation kinetics of RalGDS-YFP and RFP-RapGAP1 to the cell cortex. (D and E) Spatial localizations of RalGDS-YFP and RFP-RapGAP1 (D) or RFP-coronin (E) in chemotaxing cells. The arrow indicates the direction of movement.
GFP-RapGAP1OE cells exhibit a slightly higher speed and an increase in the number of turns, although cells move directionally toward the micropipette. However, these cells exhibit a strong defect in substratum attachment in the front of the cells (Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1). As wild-type cells move forward, the anterior of the cell protrudes off of the surface during pseudopod extension and then adheres to the substratum (Video 1; Soll, 1999; Wessels et al., 2006). In GFP-RapGAP1OE cells, an extended anterior remains off of the substratum for a longer time, during which it randomly shifts direction relative to the chemoattractant gradient, similar to myovII and vasp cells (Tuxworth et al., 2001; Han et al., 2002). Furthermore, although both wild-type and rapGAP1−/− cells exhibit small protrusions that are predominantly in the direction of the chemoattractant gradient (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1), GFP-RapGAP1OE cells produce large, rapidly extending pseudopodial protrusions that often extend in random directions relative to the chemoattractant gradient. These defects are consistent with RapGAP1 expression, as cells being unable to temporally regulate substratum attachments near the anterior of the cell immediately after pseudopod extension.

Rap1-GTP functions, in part, to control cell adhesion through the Rap1-GTP–binding Ser/Thr kinase Phg2 (Gebbie et al., 2004; Kortholt et al., 2006; Jeon et al., 2007). Phg2 localizes to the leading edge, the site of active Rap1, and helps mediate MyoII disassembly (Jeon et al., 2007). We suggest that the decrease in Rap1-GTP in RapGAP1OE cells leads to a localized increase in assembled MyoII and increased contractility, which may negatively regulate cell attachment. This localized increase in MyoII may inhibit localized F-actin assembly, which is linked to the formation of sites of cell attachment.

Subcellular localization of GFP-RapGAP1

We examined the dynamic localization of GFP-RapGAP1 in rapGAP1−/− cells. Although GFP-RapGAP1OE results in leading edge phenotypes, GFP-RapGAP1 expression complements the rapGAP1− spreading and MyoII assembly phenotypes and thus represents an appropriate reporter for observing dynamic RapGAP1 localizations.

Unstimulated cells display low levels of GFP-RapGAP1 at the cortex (Fig. 2 A). Upon uniform stimulation, cytosolic GFP-RapGAP1 transiently translocates to the cortex, and, in chemotaxing cells, RapGAP1 preferentially localizes to the leading edge, similar to Rap1-GTP (Fig. 2 A; Jeon et al., 2007). RapGAP1’s leading edge localization is consistent with its involvement in regulating adhesion at the anterior of a moving cell.

To better understand the temporal and spatial regulation of Rap1 activity by RapGAP1, we contemporaneously examined the dynamic subcellular localizations of the Rap1-GTP reporter Rap guanine nucleotide dissociation inhibitor (GDS)–YFP and RFP-RapGAP1 (Fig. 2, B–D). In response to stimulation, RapGAP1 transiently translocates to the cell cortex with a peak at ~10 s (Fig. 2, B and C). This response is ~2–4 s slower than the translocation of RalGDS–YFP (Fig. 2 C; Jeon et al., 2007). The slightly delayed RapGAP1 cortical localization relative to that of Rap1 activation suggests that the kinetics of RapGAP1 localization may provide a timing mechanism for limiting Rap1 activity. Unexpectedly, we found that the cortical region, where RalGDS accumulates in response to chemoattractant stimulation, differs slightly from that of RapGAP1. Although RalGDS always localizes immediately interior to the plasma membrane, RapGAP1 is found at the inner part of the cell cortex but partially overlapping with the domain of predominant RalGDS localization (Fig. 2 B, line scan analysis).

Fig. 2 D shows three sequential images of RalGDS–YFP and RFP-RapGAP1 localization in chemotaxing cells. 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, as shown using RFP-coronin (Fig. 2 D and E; and Videos 4 and 5, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1).

We compared the translocation kinetics of RalGDS–YFP to those in the cell cortex by chemotaxant stimulation in rapGAP1− and RapGAP1OE cells (Fig. 2 F). There was no apparent difference in the translocation kinetics of RalGDS, although at ~20 s after stimulation the level of the translocated RalGDS in rapGAP1− cells increased slightly and the level in RapGAP1OE cells decreased compared with wild-type cells. Our line scan analyses for the translocation kinetics of RalGDS in response to chemoattractant stimulation measure the highest point of fluorescence in the cell cortex. These results suggest that the kinetics of Rap1 activation at the site where Rap1 is most highly activated at the cell cortex are unaffected in this assay. However, the kinetics of Rap1 activation assayed by RalGDS pull-down are extended in rapGAP1− and greatly suppressed in RapGAP1OE cells, demonstrating that RapGAP1 regulates Rap1-GTP levels (Fig. 1 C).

We then compared the translocation kinetics of GFP-RapGAP1 to the cell cortex with those of PhaA-GFP (Funamoto et al., 2001), a pleckstrin homology domain containing PIP3 reporter, GFP-Arp3 (Insall et al., 2001), and coronin-GFP (de Hostos et al., 1991; Gerisch et al., 1995), which associates with the Arp2/3 complex on F-actin filaments and inhibits actin nucleation (Humphries et al., 2002; Rodal et al., 2005). The kinetics of GFP-RapGAP1 and coronin translocation are indistinguishable from each other and several seconds slower than those of PhaA and Arp3, which are similar to those of Rap1-GTP activation (Fig. 2 G). Thus, within the limits of our experiments, Rap1 activation correlates with actin polymerization, whereas RapGAP1 localization correlates to the termination of F-actin polymerization. RapGAP1 translocation is not regulated by phosphoinositide-3 kinase (PI3K), as it is unaffected in cells treated with LY294002 or in p3k1−/− cells (Fig. S2 B; Funamoto et al., 2002).
RapGAP1 cortical localization is dependent on F-actin and the RapGAP1 F41 domain. F-actin polymerization drives the extension of the plasma membrane at the leading edge, whereas F-actin–containing foci at the bottom of cells may function in cell–substratum adhesion (Uchida and Yumura, 2004). Fig. 3A demonstrates that GFP-RapGAP1 localizes to small structures at the bottom of the cell that also stain with TRITC-phalloidin, indicating that they contain F-actin and are therefore probably the structures described by Uchida and Yumura (2004). Consistent with the dynamic nature...
of these foci, our analyses of the structures labeled with GFP-RapGAP1 indicate they have a duration of ~20s (Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1). We did not observe RalGDS-YFP in these actin foci, which may be because of Rap1-GTP absence at these sites or reporter levels below our detection level. The examination of cells coexpressing RFP-coronin and GFP-RapGAP1 demonstrated that the two proteins exhibit the same localization to F-actin foci at the bottom of the cells and translocation kinetics similar to those of the cell cortex (Fig. 3 B), supporting earlier observations on coronin localization (Gerisch et al., 1995). Upon treatment with latrunculin A (LatA), an F-actin assembly inhibitor, the actin foci observed by GFP-RapGAP1 and coronin-GFP disappear and chemotactant-mediated GFP-RapGAP1 translocation to the cell cortex is blocked (Fig. 3, C and D). Our observations suggest that F-actin assembly is required for the cortical and foci localization of RapGAP1.

Fig. 4 (A and B) depicts the subcellular localization of a series of RapGAP1 deletion constructs and demonstrates that the N-terminal F41 domain is necessary and sufficient for RapGAP1 localization to F-actin foci, the cell cortex, and the leading edge. These localizations are all lost after LatA pretreatment, as happens with GFP-RapGAP1, suggesting that RapGAP1 translocates to the cell cortex through its N-terminal region, containing the F41 domain in a response that requires F-actin assembly. This is consistent with our findings (Fig. 2, D and E) that RapGAP1 localizes to areas enriched in F-actin.

Fig. 4 (C and D) shows that RapGAP1 deletion constructs lacking either the F41 or GAP domain do not complement the flattened morphology or the strong adhesion phenotypes of rapGAP1− cells. We investigated whether a leading edge localization of the GAP domain is sufficient to complement the rapGAP1− phenotypes by fusing the GAP domain to the N-terminal domain of PI3K1, which is necessary and sufficient for PI3K1’s plasma membrane translocation and leading edge localization. This process, like RapGAP1 localization, requires F-actin polymerization and is independent of PI3K function (Funamoto et al., 2002; Sasaki et al., 2004). Although N-terminal PI3K1-GAP localizes to the leading edge, the fusion protein does not complement the rapGAP1− cell phenotypes (unpublished data), suggesting that the F41 domain may also be required for the proper regulation of GAP function.

We identified CtxI as an F41 domain–interacting protein in a yeast two-hybrid screen (Fig. 5 A), an interaction that is specific for CtxI, as CtxII does not interact (unpublished data). Ctxs are F-actin bundling proteins that are important determinants of cell shape and regulators of cytokinesis (Faix et al., 1996, 2001; Weber et al., 1999; Faix, 2002). Fig. 5 B indicates that ctaA− or ctbB− single null strains exhibit a wild-type chemotactant-mediated GAP-RapGAP1 translocation to the cell cortex and leading edge in chemotaxing cells. However, ctaA+/B− cells exhibit a high basal level of GAP-RapGAP1 in the cell cortex and the level does not change after stimulation, a localization that requires the RapGAP1 F41 domain (unpublished data). In chemotaxing ctaA+/B− cells, RapGAP1 is found uniformly around the cell cortex, including the posterior and lateral sides of cells (Fig. 5 C). Consistent with the aberrant localization of RapGAP1 in ctaA+/B− cells, ctaA+/B− cells exhibit a reduced basal and slightly lower chemotactant-mediated Rap1 activation level compared with those of wild-type cells (Fig. S2 C) and display a slightly higher speed and an increased number of turns during chemotaxis (Fig. S2 D and Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200705068/DC1). CtxI is found in a complex with Rac1 and the IQGAPs DGAP1 and GAPA (Faix, 2002; unpublished data); however, dgap1+/gapA− cells exhibit wild-type cell RapGAP1 localization, indicating that the regulation of RapGAP1 localization by Ctx is independent of the Ctx–Rac1–IQGAP complex. RapGAP1 may interact with CtxII in CtxI’s absence, possibly in a complex with proteins other than IQGAPs. For example, DGAP1 binds to CtxII in CtxI’s absence (Faix, 2002), and ctaA+/B− cells have a level of cell attachment similar to that of wild-type cells (Fig. S2 D). One might expect cell attachment to be decreased (decreased Rap1-GTP) but, like myoII− cells, ctaA+/B− cells are very flat, and this increase in surface area may increase attachment (Jeon et al., 2007). Thus, Ctxs negatively regulate RapGAP1’s F-actin-mediated cortical localization, providing a new insight into Ctx function. We find that CtxI/II’s negative regulation of RapGAP1’s cortical localization is not general for F-actin–dependent cortical localization of signaling components, as PI3K localization is unaffected (unpublished data).

Conclusions

We show that RapGAP1, through Rap1, controls MyoII assembly (Jeon et al., 2007), which inversely correlates with cell spreading and attachment. GAP-RapGAP1+CtxI leads to decreased Rap1-GTP, defects in the ability of the leading edge of chemotaxing cells to adhere to the substratum, and an increased basal level of assembled MyoII, suggesting that MyoII assembly is inversely associated with cell adhesion (De la Roche et al., 2002; Uchida and Yumura, 2004; Jeon et al., 2007). We propose that the activation of Rap1 at the leading edge occurs simultaneously with stabilization of the protrusions by regulating cell–substratum attachments and then releasing the adhesion through deactivation of Rap1 by RapGAP1 after completion of F-actin assembly, which may be linked to Rap1’s role in controlling localized MyoII assembly. Ctx negatively regulates RapGAP1’s cortical localization, identifying a new mechanism for controlling localized responses and suggesting a new role for Ctx in regulating cell movement.

Materials and methods

Materials

We obtained LatA, LY294002, and phalloidin from Sigma-Aldrich, anti-Myc antibodies from Santa Cruz Biotechnology, Inc., monoclonal anti-pan-Ras (Ab-3) antibodies from EMD, glutathione-Sepharose beads from GE Healthcare, and nitrocellulose filters used for the adhesion assay from Millipore. Rabbit anti-Rap1 antibodies were described previously (Kang et al., 2002). Strains and plasmids

The full coding sequence of the rapGAP1 cDNA was generated by RT-PCR, cloned into the EcoRI–XhoI site of pBluescript KS (+), sequenced, and subcloned into the expression vector EXP-4[+] containing a GFP fragment. For expression of truncated RapGAP1 proteins, the various deletion rapGAP1 sequences were amplified by PCR and cloned into the EcoRI–XhoI site of an
Figure 4. Cortical localization of RapGAP1 through the F41 domain. (A) Schematic diagram of truncated RapGAP1 proteins. (B) Translocation and localization of the truncated RapGAP1 proteins. (a) Translocation kinetics of the truncated GFP-fusion RapGAP1 proteins to the cell cortex in response to cAMP stimulation. (b) Translocation of GFP-F41 to the cell cortex upon stimulation without or with treatment of the cells with LatA. (c) Localization of GFP-F41 in chemotaxing cells. *, position of micropipette containing cAMP. (d) Foci localization of GFP-F41 at the bottom of cells. Cell morphology (C) and adhesion (D) of rapGAP1⁻ cells expressing GFP-ΔF41 or GFP-ΔGAP. Experiments were performed at least three times. Error bars represent SD. Bars, 5 μm.
We made a rapGAP1 knockout construct by inserting the Blasticidin resistance cassette into a BamHI site created at nucleotide 378 of the rapGAP1 cDNA and used it for a gene replacement in the KAx-3 parental strains. Randomly selected clones were screened for a gene disruption by PCR, which we confirmed by Southern blot analysis. The constructs of GST–Rap binding domain (RBD)–RalGDS, RBD–RalGDS-YFP, and GFP-Rap1 were described previously (Jeon et al., 2007).

Assays

To assay Rap1 activation, we expressed the RBD of mammalian RalGDS 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 as described previously (Jeon et al., 2007). We performed the adhesion assays using nitrocellulose filters as described previously (Sun et al., 2003; Jeon et al., 2007).
The activated Rap1 was quantitated by densitometry and normalized with total Rap1 in at least three independent experiments. The level of activated Rap1 at 10 s after stimulation in wild-type cells was set to 1.0 and the others are relative amounts.

The Rap1 stability assay was performed as described previously (Brinkmann et al., 2002; Schultess et al., 2005) with slight modifications. A 341-aa fragment of human Rap1GAP coding for aa 75–415 was catalytically active and used for studying the mechanism of GTPase activation. We aligned the aa sequence of RapGAP1 with that of human Rap1GAP, amplified the sequence of RapGAP1 corresponding to that of the 341-aa fragment of human Rap1GAP, coding for aa 662–1055 in RapGAP1, and cloned it into the pGEX6P-1 expression vector. The GST-tagged GAP domain of RapGAP1 was expressed and purified using glutathione-coupled Sepharose beads. After extensive washing, we eluted bound proteins with phosphate-buffered saline containing 10 mM glutathione, pH 8.0. We concentrated the proteins by centrifugal concentrators (Microcon YM-10; Millipore) to 10 mg/ml, added 10% glycerol, and snap froze aliquots in liquid nitrogen and stored them at −80°C.

Cells expressing Myc-Rap1 were lysed and incubated with the GST-fusion GAP domain of RapGAP1 or GST protein without any fusion protein as a control. After incubation for 20 min, the lysates were split. One part was used for detection of GTP-bound Rap1, and the other was used for GTP-bound Ras proteins. The activated Rap1 was precipitated using GST-RalGDS, subjected to SDS-PAGE, and then visualized by Western blotting using a monoclonal antibody against the Myc tag. GTP-bound Rap1 was pulled down with the GST-Rap1-binding domain of Rap and detected by immunoblotting using monoclonal antipan-Ras antibodies (Sasaki et al., 2004). We assayed F-actin polymerization and MyoII assembly as described previously (Park et al., 2004).

Chemoattractant responses and the localization of Ras were done as described previously (Sasaki et al., 2004; Jeon et al., 2007). Images of chemotaxing cells were collected on a microscope (TE300; Nikon) with a plan Fluor lens (ELWD 40× NA 0.6; Nikon) and a camera [CoolSNAP HQ; Roper Scientific]. The frames were captured using Metamorph software (MDS Analytical Technologies) and analyzed with DIAStool (Soft Technologies, Inc.; Wessels et al., 1998).

Quantitation of membrane or cortical localization of GFP fusion proteins was done as described previously (Sasaki et al., 2004; Jeon et al., 2007). Fluorescence images were obtained using a confocal microscope (DM IRE2; Leica) with a 100× or 63×/1.4 objective and a camera (ORCA-ER; Hamamatsu) or a Dual-View Ol-11-EM–equipped camera (EM-CCD; Hamamatsu) for simultaneous imaging. Images were captured using SimplePCI software (Hamamatsu) and were analyzed using Metamorph.

Online supplemental material

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