The Dictyostelium discoideum Rap1 signalling cascade and its functions during growth and development
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER II
Three RapGEFs for multiple cellular functions during the Dictyostelium life cycle

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Part of this work was submitted to: Mol Biol Cell
Abstract

Rap1 is an important regulator of Dictyostelium cell viability, substrate adhesion, chemotaxis and morphogenesis. Despite its key role, the regulation of Rap1 activity is not well understood. Thus far, the only identified RapGEF, GbpD, has been implicated in mediating Rap1 activation during substrate attachment and in establishing cell polarity. In this report we identify two novel Rap1 specific GEFs and characterize their impact on Rap1 functions during the life cycle of Dictyostelium. GefQ and GefL proteins activate Rap1 during the vegetative growth and starvation phases, respectively. Lack of gefQ severely affects vegetative cell growth and chemotaxis in folate gradients, whereas starved gefL-null cells show decreased chemotaxis to cAMP and defects in the final stages of development. Together the three GEF proteins provide a layered mechanism of Rap1 activation for multiple cellular functions during the Dictyostelium life cycle.

Introduction

The ability to respond to intra- and extracellular signals is essential for every living organism or cell. Complicated intracellular signalling cascades, which are tightly regulated in time and space, transduce the response inside the cell (Bourne et al., 1990; Mitin et al., 2005; Wennerberg et al., 2005). Dictyostelium discoideum serves as an excellent model organism for studying signal transduction pathways involved in progression of cell division, cellular motility, chemotaxis and adhesion (Noegel and Schleicher, 2000; Wilkins and Insall, 2001). Dictyostelium combines genetic tractability and an ease of manipulation, with the added advantage that the core signalling pathways of many biological processes are conserved between Dictyostelium and higher eukaryotes (Wilkins and Insall, 2001; Muller-Taubenberger et al., 2012).

The unique life cycle of Dictyostelium allows the amoeba to respond to demands of the changing environment. During its life cycle Dictyostelium undergoes major and multiple transitions and uses a variety of signal transduction pathways. Vegetative cells actively seek bacteria by chemotaxis to folate secreted by the bacteria and then engulf them by phagocytosis (Bozzaro and Eichinger, 2011). Depletion of nutrients initiates the
multicellular stage of development. Starvation causes major changes in gene expression and cells become sensitised to cAMP which acts as strong chemoattractant for starved cells (Konijn et al., 1967). As cells migrate towards increasing concentrations of cAMP they form aggregation centres composed of as many as 100,000 cells. These large cell aggregates form migratory slugs and subsequently, by a process of morphogenesis, develop into fruiting bodies composed of dead stalk cells that support spore heads filled with dormant spores (Strmecki et al., 2005).

Small G-proteins serve as essential molecular switches in a plethora of signalling pathways and their role appears to be highly conserved among eukaryotic cells. G-proteins can rapidly shuttle between an inactive (GDP bound) and active (GTP bound) state and only the GTP bound protein can bind to downstream effectors (Bourne et al., 1991). The switch between the active and inactive state of small G-proteins is dependent on regulatory proteins. Since, small G-proteins have a very high nucleotide affinity (nM-pM range), Guanine nucleotide Exchange Factors (GEFs) are necessary for GDP/GTP exchange. GEFs facilitate the release of bound nucleotide, which subsequently allows binding of GTP that is present in excess over GDP in the cytosol (Bourne et al., 1991, Vetter and Wittinghofer, 2001). GTPase activating proteins (GAPs) stimulate the low intrinsic GTPase activity and thereby revert the conformation back to the inactive GDP-bound form (Trahey and McCormick, 1987).

*Dictyostelium* Rap1 belongs to the Ras subfamily of small G-proteins. Deletion of *rap1* appears to be lethal for *Dictyostelium* (Kang et al., 2002), suggesting an essential function for the protein. *Dictyostelium* Rap1 was first described for its role in cell morphology (Rebstein et al., 1993); however it was later shown to act in numerous other pathways, including those regulating cell growth, cell viability, response to osmotic shock, cellular adhesion, motility and multicellular development (Kang et al., 2002, Kortholt et al., 2006; Jeon et al., 2007, Jeon et al., 2009; Parkinson et al., 2009; Lee and Jeon., 2012). Despite its key role, the regulation of Rap1 remains largely unknown. So far four Rap1 specific GAPs and only one Rap1 specific GEF have been identified and characterized. RapGAP1 mediates cAMP stimulated Rap1 deactivation (Jeon et al., 2007b), both RapGAP2 and RapGAP3 function during multicellular development (Parkinson et al., 2009; Jeon et al., 2009), while RapGAP9 is linked to regulation of cell shape and cytokinesis (Mun et al., 2014). So far GbpD has been the only described Rap1 specific GEF. GbpD is involved in regulating Rap1 dependent substrate adhesion and its overexpression results in flat cells with defects in chemotaxis and polarity (Kortholt et al., 2006). Cells
lacking gbpD, still show a significant level of Rap1 activation both during vegetative growth and after cAMP stimulation (Kortholt et al., 2006), suggesting that there have to be additional Rap1 specific GEFs.

Here we show that GefL and GefQ are Rap-specific GEF proteins. Together with GbpD, these GEFs activate Rap1 in response to different stimuli, which allows regulation of various processes throughout the Dictyostelium life cycle.

Results

GEF proteins that are specific for the Ras subfamily of proteins contain a CDC25 homology and Ras exchange motif (REM). Dictyostelium contains 25 genes encoding for putative Ras-GEFs (Wilkins and Insall, 2001), of which many have been inactivated by homologous recombination (Wilkins and Insall, 2001; Kortholt and van Haastert, 2008). It is not possible to predict the specificity of these GEFs for the Rap/Ras subfamily members based on their sequences. So far only GbpD has been characterized as a Rap1 specific GEF. Previously, we showed that a multitude of the GEF proteins are involved in regulating symmetry breaking and/or confinement of Ras signalling during chemotaxis (Kortholt et al., 2013). However a subset of GEF mutants didn’t show any defect in Ras signalling, and are thus potentially GEFs for Rap1. In this report we will: (1) provide both in vitro and in vivo evidence that GefL and GefQ are Rap1-specific GEFs, (2) determine the contribution of these GEFs to folate and cAMP stimulated processes, and (3) describe the function of the three Rap1 GEFs during the vegetative state, when Rap1 is important for cell viability and substrate adhesion.

GefQ stimulates the nucleotide exchange of Rap1 in vitro

Fluorescence based nucleotide exchange can be used to determine the specificity of GEFs in vitro (Lenzen et al., 1995). In this assay G-proteins loaded with fluorescent GDP (mantGDP) are incubated in the presence of an excess of GDP. Nucleotide exchange is measured in the absence or presence of recombinant GEF protein as the decay in fluorescence, caused by the release of the fluorescent GDP from the G-protein. High
quality GefQ (910–1298AA) protein was isolated from Ecoli and its addition to fluorescent labeled Rap1 resulted in a rapid decrease in fluorescence, and thus an acceleration of the intrinsic low nucleotide exchange activity (Fig. 1A). Since previous reports suggest that GefQ is also a GEF protein for Dictyostelium RasB (Mondal et al., 2008), we performed similar exchange assays with Dictyostelium Ras proteins. Although the labeled Ras proteins that were used in the experiment are completely stable and functional (Kortholt et al., 2006), GefQ was unable to stimulate the nucleotide exchange of RasB, RasC, RasD or RasG (Fig. 1B+C), suggesting that in vitro GefQ specifically activates Rap1. Unfortunately we were unable to isolate any stable fragment comprising the catalytic GefL domain and were unable, therefore, to perform an analogous series of experiments with this protein.

Figure 1. GefQ specificity in vitro.

GEF activity was measured with excess of non-labelled GDP using mGDP-loaded (A) Rap1, (B) RasB (squares) or RasG (triangles), (C) RasC (circles) or RasD (squares) in the absence (open) or presence (closed) of 1 µM of recombinant purified GefQ. The exchange activity was measured in real time as decay in fluorescence.

GefQ and GefL mutants have impaired Rap1 activation

To test the specificity of GefL and GefQ in vivo we determined the levels of active Ras and Rap in Dictyostelium lysates using an RBD pull-down assay. Consistent with previous results (Kortholt et al., 2013), pull-down experiment with Ras-specific GST-Byr2(RBD) revealed that cells lacking gefL or gefQ have similar levels of basal and cAMP mediated RasG activation to their parental strains (Fig. 2A). To detect the amount of activated Dictyostelium Rap1 in these GEF mutants, we performed pull-down experiment
with the Rap-specific Ras Binding Domain (RBD) of human RalGDS. The amount of GTP-loaded, hence activated, Rap1 is dramatically decreased in vegetative gefQ-null cells but Rap1 activation is normal in starved gefQ-null cells (Fig. 2B). Interestingly, we observed the opposite effects for gefL-null cells with Rap1 activation barely affected in vegetative gefL-null cells but dramatically decreased in starved cells (Fig. 2C). Since gefQ and gefL-null cells exhibit normal Ras activation but defective Rap1 activation in vivo, these data suggest that GefQ and GefL are both Rap1 specific GEFs. The data also suggest that GefQ and GefL may have specific functions in vegetative and starved cells, respectively.

**GefQ and GefL mutants show aberrant spatial Rap1 activation**

Dynamic activation of Rap1 can be imaged in vivo using the previously described molecular probe – RalGDS-GFP, which specifically binds active Rap (Jeon et al., 2007a). The gbpD (Bosgraaf et al., 2005), gefL (Wilkins et al., 2005) and gefQ-null (Mondal et al., 2008) strains have been generated in a DH1, AX3 and AX2 wild-type background, respectively. Since all three wild-type control strains showed identical Rap1 activation kinetics (see also Fig 2C, Kortholt et al., 2006), we here only present the in vivo data for the AX3 parental strain.

Vegetative wild-type cells exhibit Rap1 activation in cell membrane patches and on macropinosomal cups (Fig. 3A, upper panel). Vegetative gefL and gbpD-null cells exhibit a similar pattern (Fig. 3A), consistent with the pull down data (Fig. 2B). In contrast, impaired Rap1 activation was seen in vegetative gefQ-null cells (Fig. 3A).

Upon starvation cells enter the developmental cycle and have the ability to sense and chemotaxis towards cAMP (Konijn et al., 1967). After 6 hours of starvation, aggregation competent wild-type and mutant cells were harvest in PB-buffer and random movement was recorded. Wild-type cells showed Rap1 activation at the extending pseudopods (Fig. 3A). Highly similar spatial activation of Rap1 was observed in random moving gefQ-null and gbpD-null cells. Pull-down data suggested that only gefL-null cells had lowered basal Rap1 activation in starved cells. Accordingly, we observed no Rap1 activation at the extending pseudopods in these starved gefL-null cells (Fig 3A).
Figure 2

(A) Pull-down experiment with GST-RBD(Byr2) to detect the amount of cAMP-mediated RasG activation in starved aggregation competent gefQ or gefL-null cells. Pull-down with GST-(RBD)RalGDS to detect the amount of active Rap1 in lysates of (B) vegetative wild-type, gefQ-null and gefL-null cells (C) cAMP stimulated starved aggregation competent gefQ-null cells (upper panel) and gefL-null cells (lower panel). The amount of G-protein in the pull-down fraction or total lysate was detected by western blotting, determined with α-RasG or α-Rap1 antibody. The images shown are representative for three separate experiments.

It was shown previously that the level of active Rap1 at the cell membrane increases in response to a uniform pulse of cAMP (Jeon et al., 2007a). The cAMP-mediated Rap1 response in starved gefQ-null and gbpD-null cells is highly similar to the one observed for the wild-type strain; the amount of RalGDS-GFP in the cytosol rapidly drops after stimulation, the minimum intensity is reached at 5 seconds after stimulation, and subsequently the amount of RalGDS in the cytosol returns to basal levels after approx. 30 seconds (Fig. 3B). Consistent with the pull-down activation experiment, gefL-null cells show severely impaired Rap1 activation in response to a cAMP pulse (Fig. 3B).

Together, these results and previously published data (Kortholt et al., 2006) demonstrate that Rap1 activation is regulated by at least 3 GEF proteins: GefL, GefQ and GbpD. Mutant studies reveal that GbpD and GefQ mainly regulate Rap1 activity in vegetative cells, while GefL is responsible for activating Rap1 in response to cAMP.
Figure 3. GefQ and GefL regulate dynamic Rap1 activation in vivo.

To analyse spatial activation of Rap1, the GFP-RalGDS reporter was expressed in wild-type, gefQ-null, gefL-null and gbpD-null cells. (A) Representative images of Rap1 activation vegetative (non-starved) cells (upper panel), or in randomly moving aggregation competent cells. Bar indicates 10 µm. (B) Time course of RalGDS-GFP translocation after uniform stimulation with 1 µM cAMP. Shown are the normalised intensity of cytosolic fluorescence as means and SEM of minimal 10 cells. * indicates significantly less than wild-type at P<0.01.

Role of GefQ and GefL-mediated Rap1 activation during cell migration in chemotactic gradients.

Cells expressing constitutive active Rap1, or mutants with increased levels of active Rap1, are flat and adhesive, migrate slowly and fail to properly polarize in a cAMP gradient, suggesting that Rap1 plays an important regulatory role in chemotaxis (Kortholt et al., 2006; Jeon et al., 2007a; Kortholt and van Haastert, 2008; Lee and Jeon, 2012). In addition, Rap1 is activated at the leading edge of wild-type cells moving up the cAMP gradient (Jeon et al., 2007a). However it is unclear how Rap1 is activated and how this activation contributes to efficient migration. Similarly, less is known about the contribution of Rap1 activation during chemotaxis of non-starved, vegetative cells. Here we determine which of the three Rap1 GEFs are necessary for localized Rap1 activation and efficient chemotaxis in response to folate and cAMP.

Vegetative cells are attracted to folate and other compounds secreted by bacteria, and actively seek the microorganisms by positive chemotaxis (de Wit and
Wild-type cells showed up-gradient accumulation of RalGDS-GFP (Fig. 4) and efficiently migrated (Table 1) towards increasing concentrations of folate. Both \textit{gefL}-null and \textit{gbpD}-null cells showed highly similar Rap1 activation and movement in the direction of the increasing folate gradient compared to wild-type cells (Table 1, Fig. 4). In contrast, vegetative \textit{gefQ}-null cells show reduced levels of active Rap1 at the leading edge and move with very low efficiency and speed towards a folate containing pipette (Table 1, Fig. 4).

\textbf{Figure 4. GefQ and GefL regulate dynamic Rap1 activation \textit{in vivo}.}

Representative images of wild-type and GEF mutant cells subjected to chemoattractant gradients. Cells in upper panel are vegetative cells exposed to a folate gradient, bottom panel represents starved aggregation competent cells exposed to cAMP gradient. Bar indicates 10 µm.

The three Rap1-GEFs have a completely different contribution to Rap1 activation during chemotaxis towards cAMP. Starved wild-type, \textit{gefQ}-null and \textit{gbpD}-null cells show Rap1 activation at the leading edge and move with high persistence to a pipette filled with cAMP (Table 1, Fig. 4 lower panel). In contrast, starved \textit{gefL}-null cells have hardly any Rap1 activation at the leading edge and show reduced chemotaxis, both in terms of chemotaxis index and migration speed towards cAMP (Table 1, Fig. 4).

Together our data show that Rap1 signalling downstream the folate receptor is mediated through GefQ, while signalling downstream of the cAMP receptor is mediated through GefL.
Table 1. Chemotaxis parameters of the GEF mutants and parental strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Folate (1µM)</th>
<th>cAMP (100µM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Chemotaxis Index (au)</td>
<td>Speed (µm/min)</td>
</tr>
<tr>
<td>AX2 parent</td>
<td>0.52 ± 0.16</td>
<td>10.29 ± 2.52</td>
</tr>
<tr>
<td>gefQ-null</td>
<td>0.30 ± 0.10*</td>
<td>5.17 ± 1.19*</td>
</tr>
<tr>
<td>AX3 parent</td>
<td>0.53 ± 0.16</td>
<td>7.22 ± 2.12</td>
</tr>
<tr>
<td>gefL-null</td>
<td>0.56 ± 0.21</td>
<td>7.13 ± 2.17</td>
</tr>
<tr>
<td>DH1 parent</td>
<td>0.44 ± 0.13</td>
<td>5.76 ± 1.60</td>
</tr>
<tr>
<td>gbpD-null</td>
<td>0.49 ± 0.14</td>
<td>4.35 ± 1.54**</td>
</tr>
</tbody>
</table>

Shown are the chemotaxis index and speed as the means and standard error of the means of at least 12 cells. * indicates significantly less than the parental strain at; *=p<0.01; **=p<0.05

Defective spore formation in gefL-null cells

Recent studies have shown that Rap1 plays also an important role during Dictyostelium morphogenesis by affecting cell type differentiation within the multicellular aggregate (Parkinson et al., 2009, Jeon et al., 2009). Constitutive activation of Rap1 results in breaking up of the aggregation streams and formation of aberrant tipped mound structures, due to defects in pre-stalk cell patterning. In addition, there are severe defects in slug migration (Parkinson et al., 2009). Consistently, disruption of gefL leads to severe defects in photo- and thermo-taxis (Wilkins et al., 2005). In the present studies we show that GefL is important for spore viability. While wild-type, gbpD-null, and gefQ-null fruiting bodies produce spores of normal morphology with a typical bright appearance in phase contrast microscopy, almost all gefL-null spores are smaller and of a much darker appearance (Fig. 5A). A plaque assay for spore viability on Klebsiella lawns showed that a significantly lower fraction of spores of gefL-null strain are capable of germination in comparison to the parental cell line (Fig 5B). In contrast, disruption of the gbpD or gefQ genes resulted in spores with no, or very little effect on viability (Fig 5B).
Figure 5. Spore viability is regulated by GefL.

Wild-type, gefQ-null, and gbpD-null were developed for 48 hours on NN agar and collected in PB-buffer. (A) Representative phase contrast images of the collected spores. gefL-null, but not of AX3, gefQ-null, or gbpD-null (Insets) show altered morphology. Bar indicates 10 µm. (B) The spores were plated on Klebsiella lawns and the amount of formed plaques was scored. Presented are the percentage of spores that formed plaques as the means and standard error of the means of at least three independent measurements on different days. * indicates significantly less than wild-type at P<0.01.

GefQ and GbpD are important for Rap1 signalling in vegetative cells

In addition to mediating processes that are stimulated by extracellular signals, Rap1 is also an important component of intrinsic signalling pathways. Rap1 is a major regulator of cellular adhesion; cells expressing dominant negative Rap1S17N and constitutive active Rap1G12V have reduced and increased levels of cell substratum attachment, respectively (Kortholt et al., 2006; Jeon et al., 2007a). Consistent with the role of Rap1 activation during the vegetative state, GbpD and GefQ overexpressing strains are more adhesive than the parental strains (Kortholt et al., 2006; Mondal et al., 2008), while disruption of gbpD (Bosgraaf et al., 2005) or gefQ-null resulted in cells with extremely low substrate adhesion. After one hour of shaking 80.5 ± 5% of gefQ-null cells are detached, compared to only 43.4 ± 5% of the parental AX2 strain. In contrast, the adhesion of gefL-null cells (52.6 ± 3% of loose cells) is not significantly different from the parental AX3 strain (43.5 ± 8%).
Rap1 has been implicated in the regulation of growth, cytokinesis and viability. All attempts to generate a rap1-null strain failed and knock down resulted in cells with slow growth rates and eventual cell death (Kang et al., 2002). Furthermore, Rap1 is specifically activated at the poles of the dividing Dictyostelium cells where it regulates the dynamic cytoskeletal changes needed to complete cytokinesis (Plak et al., 2014). Neither disruption of gbpD or gefL genes resulted in growth or cytokinesis defects (Bosgraaf et al., 2005; Wilkins et al., 2005). In contrast, dividing gefQ-null cells have severely reduced polar Rap1 activation and a reduced growth rate compared to the parental strain (Plak et al., 2014). We observed that addition of heat killed Klebsiella to the gefQ-null strain only partially rescued this defect by shortening the doubling time from 30 to 20 hours, suggesting a phagocytosis as well as a cytokinesis defect.

Together, the data show GefQ is a major activator of Rap1 during cell growth and that both GbpD and GefQ are important for Rap1 mediated adhesion. In contrast, GefL does not appear to have a major function during intrinsic regulated processes in vegetative cells.

**Discussion**

Rap1 appears to be an essential protein that performs a variety of functions during the Dictyostelium lifecycle (Kang et al., 2002; Jeon et al., 2007; Lee and Jeon, 2012). Here we showed that at least three independent GEF proteins, GbpD, GefL and GefQ, are responsible for activating Rap1, and these three proteins function during various stages of Dictyostelium development (Table 2).

GbpD is primarily responsible for regulating Rap1 dependent substrate attachment (Bosgraaf et al., 2005; Kortholt et al., 2006). Thus, cells lacking gbpD have severely reduced adhesion strength during vegetative growth and also during the starvation process and chemotaxis to cAMP (Kortholt et al., 2006).

GefQ also contributes to Rap1-mediated substrate attachment, although to lesser extent than GbpD, and is in addition, important for folate chemotaxis. Cells lacking gefQ exhibit drastically reduced basal and folate stimulated Rap1 activation. GefQ was previously shown to be localized to sites rich in actin filaments (Mondal et al., 2008),
suggesting it may be part of a basal feedback loop, between actin and Rap1 signalling. Interestingly, the in vitro exchange assay revealed that GefQ specifically activates Rap1 and not any of the Ras proteins. This is contrary to a previous report that, concluded that GefQ acts as an exchange factor for RasB (Mondal et al., 2008). Since this earlier conclusion was based on in vivo pull-down experiments, it is possible that GefQ affect RasB activity indirectly via proteins that are activated downstream of Rap1. Cells lacking gefQ have defects during both the vegetative growth and multicellular development (Mondal et al., 2008). Vegetative cells have normal levels of active RasB (Mondal et al., 2008) and severely impaired Rap1 activation levels (this study), while starved cells have normal levels of Rap1 activation (this study) and strong decreased RasB activity (Mondal et al., 2008). This suggests that in vegetative cells GefQ primarily activates Rap1 and in starved cells may have exchange activity for RasB. Thus, we would like to suggest that GefQ is a Rap/Ras dual specific GEF, which, dependent on the protein conformation, can activate either RasB or Rap1. Dual specificity Rap/Ras GEF proteins have been described before: CalDAGGEF1 is a cytosolic GEF for Rap1, however Ras-GRP2, a protein produced from alternatively spliced CalDAGGEF1 gene shows different localization and specificity to Ki-Ras, N-ras, Rap but not Ha-Ras (Clyde-Smith et al., 2000). Consistent with such a mechanism, two forms of GefQ proteins have been identified at different developmental time points (Mondal et al., 2008). Alternatively, the switch between Rap/Ras specificity of GefQ, may be mediated by phosphorylation of the protein. Recently a phospho-proteomic screen showed that GefQ is rapidly phosphorylated in response to chemoattractant stimulation (Charest et al., 2010).

<table>
<thead>
<tr>
<th>GEF</th>
<th>Vegetative state</th>
<th>Starvation</th>
<th>Morphogenesis</th>
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<tr>
<td></td>
<td>Growth rates</td>
<td>Adhesion</td>
<td>Folate chemotaxis</td>
</tr>
<tr>
<td>GefQ</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GbpD</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>GefL</td>
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</table>

Table 2. Biological functions of the three Rap1 GEFs during Dictyostelium life cycle.  
* (Mondal et al., 2008)  
** (Wilkins et al., 2005)  

GefL makes no contribution to Rap1 activation during the vegetative state, but is important during development. Previously it has been shown that cAMP mediated Rap1 activation depends on RasC/RasG (Bolourani et al., 2008). *gefL*-null cells have severely defective cAMP-mediated Rap activation, but show normal kinetics of Ras activation (Kortholt et al., 2013), suggesting that GefL may be the missing link between Ras and Rap signalling in *Dictyostelium*. Cells lacking *gefL* showed a decreased chemotaxis index and a decreased motility towards the cAMP source, confirming previous reports that Rap1 activation contributes to efficient cAMP chemotaxis. GefL is also important during multicellular development. *gefL*-null cells are deficient in slug photo- and thermotaxis (Wilkins et al., 2005), and after the seemingly normal culmination process, the mutant strain produces spores with damaged spore coats and very low viability. The prominent role of *gefL* at later stages is most likely due to its expression profile: very low expression during the first hours of development, which increases after 4 hours of starvation reaching its maximum expression levels during multicellular stage of the life cycle (Rot et al., 2009).

In summary, four GAP proteins and three GEFs regulate Rap1 activity: GbpD, GefQ and RapGap9 regulate Rap1 activity during vegetative growth, while GefL, GbpD, RapGap1, RapGap2 and RapGap3 are needed for cAMP regulated processes of chemotaxis and multicellular development. Although, the regulation of these GEFs and GAPs is not completely understood yet, transcription, subcellular localization, and posttranslational modifications most likely all play a role. Together this complex network allows Rap-mediated cytoskeletal changes in response to different intra- and extracellular stimuli which is important for many cellular processes.

Materials and Methods

**DNA constructs, strains and cell culture**

*gefQ* was amplified by means of PCR, and subsequently ligated into the BglII and SpeI sites of a pDM310 inducible *Dictyostelium* expression plasmid system (Veltman et al., 2009). *Dictyostelium* cells were grown in HL5-C media (Formedium) on nunclon-coated dishes or in Erlenmeyer flasks. For growth of mutant strains, the medium was
supplemented with the appropriate antibiotics; 10mg/ml G418, 50mg/ml hygromycin B or 10mg/ml Blasticidin S. The previously described *gbpD* null (Bosgraaf *et al.*, 2005), *gefQ* null (Mondal *et al.*, 2008) and *gefL* null (Wilkins *et al.*, 2005) were obtained from the *Dictyostelium* stock center (Fey *et al.*, 2012)). For starvation, *Dictyostelium* cells were harvested, washed with Phosphate Buffer (PB) (10 mM KH$_2$PO$_4$/Na$_2$HPO$_4$, pH 6.5), and plated on Non-Nutrient Agar plates (1.5% Agar in PB). After 6 hours of starvation, aggregation-competent cells were harvested in PB at a density of 6 x 106 cells per ml.

**Rap1 activation assay**

Rap1 activation assays were performed as described previously (Bolourani *et al.*, 2008). *Dictyostelium* cells were resuspended in buffer and an equal volume of Lysis buffer (20 mM Na$_2$HPO$_4$, pH 7.2, 2% Triton X-100, 20% glycerol, 300 mM NaCl, 20 mM MgCl$_2$, 2mM EDTA, 2mM Na$_3$VO$_4$, 10mM NaF, Roche protease inhibitor tablets) was added. The cell lysates were pre-cleared by centrifugation, 10 min, 4°C, 14000g, and protein concentration in supernatant samples was measured with the Bradford assay. 400µg of proteins was mixed with 100µg of recombinant purified GST-RBD(Byr2) or GST-RBD(RalGDS) (Kortholt *et al.*, 2006). The samples were incubated with glutathione-sepharose beads (GE-Healthcare) for 1 hour at 4°C and subsequently washed 3 times with lysis buffer. Bound proteins were eluted by boiling in 1xSDS buffer and resolved on SDS-page gels. The amount of activated Rap1 or RasG was visualised by western Blot with primary αRap1 or αRasG antibody (Bolourani *et al.*, 2008).

**Microscopy**

Live *Dictyostelium* cells with RalGDS-GFP marker (Kortholt *et al.*, 2006) were observed using a confocal laser scanning microscope (LSM 510 META-NLO; Carl Zeiss Microimaging, Inc.) equipped with a 63x/NA 1.4 objective (Plan-Apochromatic; Carl Zeiss Microimaging, Inc.). The fluorochrome GFP (S65T variant), was excited with a 488-nm argon/krypton laser. The fluorescence was filtered through a BP500-530 IR and a LP560
filter, and was detected by a photomultiplier tube. Images were analysed using ImageJ software (Abramoff et al., 2004).

Chemotaxis was tested with the previously described micropipette assay (Kortholt et al., 2011). Shortly, for folate chemotaxis vegetative cells were washed in PB buffer and subjected to a gradient of 1µM folate released from a micropipette (tip opening 3µm, 4hPa pressure). For the cAMP chemotaxis assay aggregation competent cells were subjected to the cAMP gradient released from micropipette filled with 100µM cAMP solution (tip opening of 0,5µm, 0hPa pressure). Chemotaxis was monitored with an inverted light microscope and images were recorded every 10 seconds. Chemotaxis index and speed was analysed using ImageJ software as described previously (Kortholt et al., 2011; van Haastert et al., 2007).

**Adhesion assays**

To determine the strength of cellular attachment, we used a previously published protocol (Bosgraaf et al., 2005). Briefly, cells were grown overnight in a six-well plates (Nunc) to a maximum of 70% confluence. The medium was replaced and cells were incubated on rotary shaker for 1 hour at speed of 150 rpm. Subsequently, the fractions of adhesive and loose cells were determined in triplicate in Thoma-chambers. Each experiment was performed at least three times.

**Spore viability assay**

*Dictyostelium* cells were allowed to develop for 48 hours on NN agar plates and spores were collected in PB buffer. Spores were counted in Thoma chambers and 100 spores were plated on 1/3 SM plates with *Klebsiella* lawns. The plates were incubated for 96 hours at room temperature and the amount of plaques was scored.
**GEF exchange assay**

The GEF domain of GefQ (910–1298) was cloned into the BamHI site of pGEX 4T3 (GE Heathcare). The GST-GEF domain was expressed in *E.coli* and subsequently purified by GSH affinity and size exclusion chromatography (SEC). The indicated small G-proteins were purified as described before (Kortholt *et al.*, 2006). The small G-proteins were loaded with the fluorescent analogue mantGDP, 2’-/3’-O-(N’-methylantraniloyl)-guanosine-diphosphate, by incubating them in the presence of 10mM EDTA and a 20-fold excess of mantGDP for 2 h at room temperature. Unbound nucleotide was removed by SEC. The fluorescent loaded proteins were incubated at 25°C in assay buffer (50 mM Tris-HCl, pH 7.5, 5mM MgCl₂, 50 mM NaCl and 5 mM DTE) and the exchange reaction was started by adding a 200 fold excess of unlabelled GDP. Experiments were performed in the presence or absence of GefQ GEF. The nucleotide exchange was measured in real time as decay in fluorescence using a Spex spectrofluorometer (Spex Industries), with excitation and emission wavelength of 366 and 450 nm, respectively.

**References**


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