Symmetry breaking in navigating cells
Kataria, Rama

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Chapter IV
A Proteomic Approach to identify the networks regulating chemotaxis

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Abstract
One of the major challenges in cell biology is to understand the molecular mechanisms by which a small extracellular gradient of chemoattractant generates a steep intracellular gradient of signaling molecules. This symmetry breaking in signaling is essential to enable the cell to migrate along a chemical gradient. This process, called chemotaxis, is highly complex and involves numerous signaling molecules and interconnected pathways. Previously, we have identified a basal signaling module consisting of heterotrimeric and monomeric G-proteins that is sufficient to induce Dictyostelium chemotaxis. Here we used a highly sensitive mass-pull-down proteomic strategy to identify further components of this basal signaling pathway. We used ten different bait proteins and found approximately 1000 interacting partners, and several of them were previously unknown. With our data we identified new proteins involved in regulation of heterotrimeric G-protein signaling, and some candidates which might serve as putative links between the heterotrimeric and monomeric G-protein signaling. Moreover, we also identified a number of proteins that might establish the direct link between heterotrimeric G-proteins and regulators of cytoskeleton. This approach together with the advantages of Dictyostelium as a model system gives new insights in the molecular mechanisms underlying the regulation of G-protein signaling and cytoskeleton during chemotaxis.
Introduction

Chemotaxis, the directional movement of cells towards a chemical gradient, is a fundamentally important process for both prokaryotes and eukaryotes (1). In prokaryotes chemotaxis is necessary to find nutrients or escape from toxins (2), while in metazoans chemotaxis is important for tissue morphogenesis, immune response, and embryonic organization (3–5). Since chemotaxis is involved in such essential processes, minor defects in chemotaxis can cause the onset of various diseases including asthma, cancer, arthritis, cardiovascular diseases and inflammatory bowel diseases (6–8). It is thus of great interest to understand the molecular pathways regulating chemotaxis.

The most commonly used model systems for studying chemotaxis are the slime mold Dictyostelium discoideum and mammalian neutrophils (9, 10). Chemotaxis is essential for the Dictyostelium life cycle: during the vegetative stage, cells chemotax towards folate secreted by bacteria upon which they feed. Starvation induces a dramatic change in gene expression and cells start to produce and secrete cAMP. Simultaneously cells sense and chemotax towards the cAMP secreted by neighboring cells (11), resulting in the formation of multicellular aggregates (11). By the process of differentiation and morphogenesis these aggregates develop within 24 hours of starvation into fruiting bodies, consisting of a spore head mounted on top of an elongated stalk of cellulose-encased dead vacuolated cells (12).

Dictyostelium cells are highly sensitive to gradients of cAMP and folate and exhibit strong chemotaxis in very shallow concentration gradients (~2% across their body length) (13). A central problem is to understand how such a small extracellular gradient generates a steep gradient of intracellular signaling components leading to cytoskeleton rearrangement and cell migration. Previously we have identified the minimal requirements for chemotaxis and proposed a basal signaling network (14). A gradient is detected by binding of ligands to G-protein coupled receptors (GPCRs), which activates the associated heterotrimeric G-proteins (15), and subsequently results in Ras activation at the side of the cell facing the gradient (16). Receptor occupancy and activation of heterotrimeric G-protein is proportional to the steepness of the extracellular gradient of chemoattractant (17, 18), while Ras activation at the leading edge is higher than the steepness of the extracellular gradient (19, 20). This suggests that amplification of the extracellular signal and symmetry breaking occurs between heterotrimeric G-proteins and Ras proteins. Interestingly, heterotrimeric G-protein subunits play different roles in Ras activation; \(\text{G}^{\beta\gamma}\)-subunit is essential for the initial uniform Ras response, while the \(\text{G}^{\alpha}\)-subunit is necessary for symmetry breaking (21). Symmetry breaking in Ras signaling and its downstream effectors is sufficient for F-actin polymerization at the
leading edge and directional movement in steep gradients of chemoattractant (14). Only in shallow gradients, the signaling enzymes, PI3K, TorC2, PLA2 and sGC, are necessary for chemotaxis to amplify the cAMP signal. The important questions that rose while deducing the basal signaling network, concern the identification of the proteins regulating the heterotrimeric G-protein cycle, and the characterization of the connecting links between heterotrimeric and monomeric G-proteins. Also, how symmetry breaking in G-protein signaling is linked to cytoskeleton dynamics and subsequently cell migration is not known yet (Fig 1A). In this chapter we describe a proteomic based strategy that was used to identify new components of the basal signaling pathway that partly answers these questions. The detailed characterization of two components, GxcC (22), Ric8 (23; Chapter 5) and LrrA (Chapter 6) have been described. Here we describe how these and other components, including RasGEFs, RacGEFs, Rac and, SCAR/WAVE form a complex hierarchy of interacting networks. Together our data give new important insights into the mechanisms essential for regulation and activation of heterotrimeric and monomeric G-proteins and actin-myosin cytoskeleton during chemotaxis.

Results and Discussions

Proteomic Approach

Our model for the minimal chemotaxis pathway can serve as a framework to understand the basic mechanism of chemotaxis (14), however it is essential to identify further components of the basal signaling pathway (Fig 1A). We therefore adopted a mass pull-down and mass spectrometry based proteomic approach (22, 23) (Fig 1B): GST fused recombinant $G_{\beta\gamma}$, $G_\alpha$-subunits, and monomeric G-proteins (Table 1) were immobilized on a GSH column and incubated overnight with Dictyostelium cell lysate. The column was washed and the bait and binding partners (interactome) were subsequently eluted and subjected to LC-MS/MS analysis. Protein identifications were based on the presence of at least two unique peptides found in the MS/MS screen. The cut-off for confidence of identification probability for the identified peptides was set at 95%. To allow comparison of the different samples, six housekeeping proteins, namely, DSC-C1, ATP5B, ABPA (or ACTNA), ABPC (or GELA), MDHB, and GPDA (or G3P), were used for normalization. The Z-score or normalized count, for each
**Figure 1** (A) Basal chemotaxis pathway. The heterotrimeric G-protein complex is activated upon binding of chemoattractant to cell surface receptors which provides prolonged activation of Ras and F-actin at the leading edge of the cell, thereby inducing chemotaxis. To understand the model completely it is essential to identify the regulators and downstream effectors of Gαi and Ras proteins. (B) Work Flow. The recombinant GST-tagged proteins were purified by affinity chromatography and incubate with *Dictyostelium* cell lysate overnight. The unbound proteins were washed away and bait proteins with their binding partners were collected. The proteins were separated on SDS-PAGE and analyzed with mass spectrometry.
Table 1: The numbers in red represents the total number of proteins identified in the screen of the specific bait protein indicated and the number in the last column (blue) lists the unique proteins identified specifically in the bait protein indicated. The number in black represents the number of proteins that are also found in the screen with the other bait proteins. Thus in the first line, number 146 represents the number of proteins identified in the screen of Go1, of which 4 proteins were also found in the screen with Go2_starved.

The protein was calculated on the basis of its spectral count and its average count in the particular bait (see Materials and Methods). These normalized counts were used to detect the total number of identified proteins, and to determine the overlap between the whole interactome of the different baits (Table 1, Fig 2A and 2B). There were at least 50 to 100 hits per bait protein, and several of them were uniquely identified to be interacting specifically with that respective bait protein (Table 1). In total there were approximately 450 unique proteins identified which when compared with the entire proteome of Dictyostelium (~13,000) is

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<th>Go8_GDP</th>
<th>Go8_GppNHp</th>
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substantially less, indicating that these interactions are specific. Surprisingly, the Ga2-subunit screen with lysate of starved cells, and RasG screen with vegetative cell lysate resulted in comparatively less number of hits (Table 1). This might indicate the highly specific roles of these proteins resulting in them interacting with very less proteins. On the basis of overrepresented GO terms in comparison to the entire proteome, each protein dataset thus obtained was characterized in terms of molecular function, cellular localization and possible biological functions. The results are presented as heat maps for better visualization and efficient interpretation of the experimental results, which reveal several important general conclusions (Fig 3). First, we could conclude that many Ga-subunit interacting proteins are localized at the cytoskeleton, more specifically at the actin cytoskeleton, whereas the Gβγ interacting proteins are not. Moreover, the interactome of Ga-subunits and not of Gβγ, are involved in cell morphogenesis. The heat maps also showed that the interactome of nearly all Ga-subunits tested are involved in chemotaxis, except Ga8, but not in thermotaxis and phototaxis except Ga1 which is known to be involved in phototaxis (24). Additionally, Ga1 and Ga4 are also involved in cell cycle regulation and endocytosis. The interactomes of small G-protein interacting partners are involved in cell cycle regulation, chemotaxis, phototaxis and thermotaxis, with the only exception of RasB interacting members which do not seem to play any role in thermotaxis. The proteins interacting with monomeric G-proteins are localizing majorly at the pseudopod, specifically at the actin cytoskeleton, and in addition RasB interacting partners also localize at the nuclear matrix and filopodium.

Several specific hits obtained from the MS screen were analyzed in more detail as described below. Finally, we used these data to get more insight in the mechanism by which:

1. the heterotrimeric G-protein cycle is regulated
2. heterotrimeric G-proteins induce symmetry breaking in Ras signaling, and
3. symmetry breaking in G-protein signaling induces cytoskeleton rearrangements and subsequently cell migration.

How is the heterotrimeric G-protein cycle regulated?

Dictyostelium has one Gβ, one Gγ and 12 Ga units (25–27). Gβγ is essential for chemotaxis and development (14, 25). Ga2 and Ga4 are essential for chemotaxis towards cAMP and folate, respectively (28, 29). In contrast, Ga1 and Ga8 don’t have a major role during chemotaxis; Ga1 is implicated in regulation of cGMP pathway, cytokinesis, phospholipase activity, negative regulation of CMF (conditioned medium factor) signaling, prestalk zone...
Figure 2 (A) Cluster analysis. The bait proteins were clustered on the basis of Z-score to determine the extent to which the various baits bind to the same protein. (B) RckA, Ric8, and Roco10 were identified on the basis of their Spectral Count (total number of spectra identifying peptides of that specific protein).

formation (28, 30–32), while Gα8 is involved in cell proliferation, adhesion, and cell differentiation (33–35). The previous studies suggest that Gα2 and Gα4 use similar pathways to mediate chemotaxis (28, 29). Interestingly, despite serving different functions all G-proteins cluster together in the dendrogram (Fig 2A). The conventional heterotrimeric G-protein cycle starts by binding of ligand to the GPCR, which leads to nucleotide exchange on the Gα-subunit thereby converting it to the active Gα-GTP form. Subsequently, the heterotrimeric complex dissociates and both Gβγ and Gα-GTP transduce the signal to their downstream effectors (16, 36–38). The signal is terminated by hydrolysis of Gα-bound GTP by intrinsic GAP activity of Gα subunits assisted by RGS proteins (Regulators of G-protein Signaling) (39). Dictyostelium contains 8 RGS proteins, however their function is not well
Figure 3 Heat Maps describing the interactome obtained from MS screen, on the basis of overrepresented Go terms (left side) in each condition (bottom). Dark colour indicates the GO term mentioned on the left is highly represented in the interactome of the specified bait protein, and light colour means it is less represented. The value represents the P-value for the Fisher Exact test.
understood (40). We identified RckA, a RGS domain containing kinase, as a specific binding partner of \( \text{G} \alpha_2 \) (Fig 2B). Recently it has been shown that \( rck4 \)-null cells have faster chemotaxis whereas the overexpressor mutant has a reduced chemotaxis speed, which would be consistent with a function as GAP for \( \text{G} \alpha_2 \) (41).

In the conventional canonical cycle only GPCRs function as GEFs for \( \text{G} \alpha \) proteins, however accumulating evidence suggest that also non-canonical activation of \( \text{G} \alpha \) is important for its function (42–44). We identified Ric8 as a non-receptor GEF for \( \text{G} \alpha_2 \) and \( \text{G} \alpha_4 \) (Fig 2B) (23) (Chapter 5). Ric8 competes with \( \text{G} \beta \gamma \) to bind free \( \text{G} \alpha \)-GDP, and converts it back to the active \( \text{G} \alpha \)-GTP form (23). It thereby amplifies and extends the G-protein signal, which is essential for \( \text{G} \alpha \)-mediated symmetry breaking of Ras during chemotaxis and development. Mammalian Ric8, like \( \text{Dictyostelium} \) Ric8, acts as a GEF for \( \text{G} \alpha \) proteins, but in addition also functions as a molecular chaperone, which is important for folding of nascent \( \text{G} \alpha \) subunits and their association with cell membranes (43, 45–47). However, in our experiments we observed that we had similar steady state expression of \( \text{G} \alpha_2 \) which was functional and coupled to the receptor even in the absence of Ric8. Moreover, the amount of \( \text{G} \alpha_2 \beta \gamma \) complex coupled to the receptor, detected by FRET change between \( \text{G} \alpha_2 \)-CFP and \( \text{G} \beta \gamma \)-YFP was also unchanged in \( \text{ric8} \)-null cells. Thus in our studies we did not find any evidence of Ric8 acting as a chaperone for \( \text{G} \alpha_2 \) (Chapter 5b) (48).

Both in mammals and in \( \text{Dictyostelium} \) the regulation of Ric8 activity is not well understood (49). It has been suggested that in human, RGS14 integrates conventional \( \text{G} \alpha_1 \) and Ric8A signaling (50). Interestingly, we identified the RGS containing protein Roco10 as binding partner of \( \text{Dictyostelium} \) Ric8 (Fig 2B). Initial studies showed that Roco10 indeed binds to \( \text{G} \alpha_2 \) via its RGS domain (data not shown). Studies addressing the function of Roco10 to Ric8 are currently still in progress, however it is tempting to speculate that, in analogy to human RGS14, Roco10 functions as an integrator of Ric8 and \( \text{G} \alpha \) signaling. The interesting hypothesis would be that immediately after hydrolysis of \( \text{G} \alpha \)-GTP, Roco10 binds to \( \text{G} \alpha \)-GDP thereby preventing it from binding to \( \text{G} \beta \gamma \). This in turn, facilitates the binding of Ric8 to \( \text{G} \alpha \)-GDP which can then convert it back to the active form and thus amplify the heterotrimeric G-protein cycle (Fig 4).

How do heterotrimeric G-proteins induce symmetry breaking in small G-protein signaling?

Activation and dissociation of the heterotrimeric G-protein complex results in transient activation of Ras proteins (51); \( \text{G} \beta \gamma \) is essential for initial uniform Ras activation, while the
Gα-subunit is essential for Ras activation at the leading edge (21). Ras, as well as Rac (see below), belong to the family of small G-proteins (52). Although the Ras family members have a high sequence identity (>44.9 %) they have clearly distinct roles (20). Previous studies have shown that activation of the small G-proteins RasC and RasG is essential for chemotaxis and development (20, 53, 54), while RasB is an important regulator of the cell cycle and cell proliferation (55, 56). Like heterotrimeric G-proteins, the three Ras proteins tested (RasB, RasC and, RasG) cluster in one branch despite having distinct functions (Fig 2A). The small G-protein family also functions as molecular switch (57). Small G-protein cycle is strictly regulated by RasGEFs that catalyze the intrinsic slow exchange of GDP to GTP and RasGAPs that stimulate GTP hydrolysis thereby converting it back to the inactive GDP-bound form (58). Dictyostelium contains 25 RasGEFs and 17 RasGAPs, of which only a few have been characterized to some extent (59–61). The local activation of specific GEFs and GAPs results in spatial and temporal activation of Ras confining it to the leading edge (19, 21, 59). RasGEFR has been identified to specifically regulate the activation of RasG, and was also found as its interacting partner in the screen (60). Interestingly, we identified GEFF as binding partner of both Gα and Ras proteins. All attempts to make gefF-null have failed so far, suggesting it is an essential gene (59). GEFF contains, in addition to the catalytic GEF domain, three tandem kelch repeats which are predicted to bind to the actin cytoskeleton (59). This could thus suggest that RasGEFF, in addition to transducing the signal directly from heterotrimeric G-proteins to Ras proteins also may provide a feedback loop from the actin cytoskeleton back to the activation of small G-protein signaling (Fig 4). Another interesting hit that could be a potential link between heterotrimeric and monomeric G-proteins is a leucine rich repeat protein LrrA, found in the screen of both Gα and Ras proteins. Previous studies have shown that LrrA is essential for development and might be involved in cytoskeleton remodeling (62). Our studies suggest that LrrA is a scaffold protein that connects heterotrimeric and monomeric G-proteins. As both knock-out and overexpression of LrrA results in altered Ras activation, the protein might play an important role in Ras activation and symmetry breaking (Chapter 6).
Figure 4 Regulators of heterotrimeric G-protein cycle and symmetry breaking in Ras Signaling. Upon activation, the heterotrimeric G-proteins are dissociated into Go-GTP and Gβγ. Due to intrinsic GTPase activity of Go, the bound GTP is hydrolyzed into GDP. This process is assisted by RGS protein, RckA. Ric8 binds to the dissociated GoGDP and facilitate its conversion back to the active Go-GTP form, thus acting as a non-receptor GEF. Roco10 was identified in Ric8 screen and might integrate the non-canonical Go-Ric8 signaling. RasGEFF, found in the screens with Go-subunits and RasC as bait, might be transducing the signal from Go-GTP to Ras and thereby activating it. LrrA (green), found in the screens with Go, RasB, and Rac1 as bait, acts as a scaffold during signal transduction from heterotrimeric proteins to Ras protein. New identified links are shown in blue and green.
How does symmetry breaking in G-protein signaling influence cytoskeletal dynamics?

Symmetry breaking in Ras signaling is sufficient to induce major rearrangement of the actin and myosin cytoskeleton (14). At the leading edge, polymerization of F-actin induces the formation of pseudopodia, while simultaneously the acto-myosin filaments formed at the side and at the rear of the cell inhibit pseudopod formation and are involved in retraction of the uropod (1). Several groups of proteins have been implicated in this process, including heterotrimeric and monomeric G-proteins, SCAR, ARP2/3 and the four amplification pathways, TORC2, PI3K, sGC and PLA2 (14, 20, 63–65). However, the exact mechanism by which G-protein signaling regulates actin/myosin rearrangement remained largely unknown (14).

The best studied Ras signaling pathways, PI3K and TORC2, have been implicated in actin polymerization at the leading edge of chemotactic cells (66–69). Although both pathways are not essential for chemotaxis they provide amplification of extracellular signal by increasing the sensitivity for chemotaxis in shallow gradients (14). Both PI3K and TORC2 have also been shown to play a minor role in confinement of active Ras at the leading edge (21). TORC2 is a tetrameric complex comprising of TorA, Pia, Rip3 and Lst8, and has been previously suggested to integrate signaling pathways regulating aggregation (70). The proteomic screen revealed that each component of TORC2 complex is interacting with different heterotrimeric or monomeric G-protein (Table 2). Lst8 was found specifically in the MS screen of Gα2, Gβγ in consistence with previous observation (70), and interestingly with the screen of Rac, which is novel. Rip3 which is important for chemotaxis and cAMP relay and was identified as a Ras-interacting protein (71) was found in the MS screen of RasG in consistency with literature. Also RapA was found to be a novel interacting partner of Rip3 (Thesis Khanna A). Similarly TorA was found only in RasC screen and Pia interacts specifically with Gβγ. Thus our data provides strong evidence that suggest the differential regulation of TORC2 complex at multiple levels by different G-proteins in response to distinct stimuli resulting in cytoskeleton rearrangement during cell migration (Thesis A. Khanna).

Rap1 activation at the leading edge occurs downstream of RasG (73). Recently, we could show that GefL is essential for cAMP mediated Rap1 activation, suggesting that RasG is activating Rap1 via GefL (Thesis Plak K). Rap1 regulates adhesion and myosin disassembly at the leading edge via GbpD, a RapGEF and a serine/threonine kinase Phg2 (74–76), and Rap1 regulates actin polymerization via activation of PI3K (77). Rap1 is also involved in activation of small G-proteins of the Rac family (22, 74, 75, 78), which are the
major regulators of cytoskeleton proteins, mainly involved in polymerization of F-actin at the leading edge (79, 80). Interestingly, in our cluster analysis, the proteins that interact with Rac, clusters together with the proteins interacting with heterotrimeric G-proteins (Fig 2A) rather than with Ras proteins. This suggests that G-proteins might directly activate Rac1/actin, which is contradictory to present view in literature suggesting that regulation of Rac activity occurs downstream of Ras (20, 81, 82). Our current hypothesis that heterotrimeric G-proteins are directly involved in regulation of Rac activation is also strongly supported by the observation that as the RacGEFs: DocA, DocB, DocD, and ZizB, were found to be interacting with Gα-subunits (Fig 5). Rac activates the pentameric SCAR/WAVE complex, comprising of ScrA, PirA, AbiA, NapA, and Hspc300, by interacting with PirA (63, 83, 84). The SCAR/WAVE complex acts as signaling hub and plays a critical role in regulating actin dynamics (65, 72, 84, 85). The regulation of activity of SCAR/WAVE complex and function of its individual component is still not well understood (63). Interestingly, our MS screen revealed that each component of this multi-protein complex interacts with different G-proteins suggesting that it is regulated at multiple levels (Table 2). ScrA was identified in the MS screen of Gα2, Gα4 and, RasB. Other important part of the SCAR complex, PirA which has been known to regulate actin dynamics by regulating SCAR activity and to bind directly to Rac (72) was found to be interacting with all tested Gα-proteins, RasB, RasC and Rac1. NapA was found in MS screen of Gα, Gβγ, RasB, RasC and Rac1. Thus the SCAR complex is integrating the signals from multiple entities as each of the components is regulated separately by different G-proteins. Activated SCAR/WAVE complex leads to the recruitment and activation of Arp2/3 via ScrA, which results in actin-nucleation and pseudopod formation (86, 87). All evidence gathered from the MS data shows that Gα proteins are directly interacting with regulators of actin cytoskeleton suggesting that Gα acts as the activator of signaling cascade which results in polymerization of F-actin at the leading edge.
Table 2: Multi-protein signaling complexes are regulated by different G-proteins. In the screens with the proteins as bait different components of the TORC2 or SCAR/WAVE complex were found. Thus TorA was found in a screen with RasC but not in other screens. All components of the TORC2 and SCAR/WAVE complex were found in screens with at least one bait, except AbiA and Hspc300, two components of the SCAR/WAVE complex, that were never found.

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<td>RasG, RapA</td>
<td>Rip3</td>
<td>(71) (Thesis Khanna A)</td>
</tr>
<tr>
<td>Gα2, Gβ, Rac</td>
<td>Lst8</td>
<td>(70)</td>
</tr>
<tr>
<td>Gβγ</td>
<td>Pia</td>
<td></td>
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<tr>
<td>Gα1, Gα2, RasB</td>
<td>ScrA</td>
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<tr>
<td>Gα1,2,4,8, RasB, C, Rac1</td>
<td>PirA</td>
<td>(72)</td>
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<tr>
<td>Gα1,2,4,8, Gβγ, RasB, C, Rac1</td>
<td>NapA</td>
<td></td>
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<tr>
<td>Not found in screens:</td>
<td>AbiA</td>
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<tr>
<td></td>
<td>Hspc300</td>
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**Figure 5 G-protein signaling influence cytoskeletal dynamics.** Upon stimulation, the Gα-subunit functions as a local activator leading to polymerization of F-actin at the front (black); whereas Gβγ is involved in regulating the pathways which inhibit the extension of pseudopods at the lateral sides and at the back of the cell (green). Thus Gβγ acts as a global inhibitor. IQGAP-cortexillin complex majorly works at the sides where it inhibits the polymerization of F-actin and promotes the assembly of Myosin filaments (red). Dotted lines indicate the new connections identified in MS screen. The area in gray represents the basal pathway downstream of heterotrimeric G-proteins.
However, regulation of the cytoskeleton at the sides and at the back of the cell is as important as at the leading edge. IQGAPs are involved in regulation of actin-myosin dynamics at the side of the cell by binding to Rac1-GTP (88, 89) and along with cortexillins, forms a quaternary complex consisting of Rac1GTP-GAPA-CtxA-CtxB (88, 90). This complex, localized at the back and the side of a chemotactic cell, interacts with actin cytoskeleton and regulates cortical dynamics. Furthermore, localization and disruption studies done with cortexillin mutants also suggests that these proteins are involved in inhibition of lateral pseudopod extension (91, 92). As expected, IQGAP2 (GAPA) and both cortexillins were identified in the Rac1 interactome. Interestingly our proteomic data revealed additional interaction of the quaternary IQGAP complex with Gα, Gβγ-subunits, and Ras proteins, suggesting G-proteins are also involved in regulation of actin cytoskeleton at the sides and at the back of the cell.

The extension of pseudopods at the sides and at the back of the cell is majorly inhibited by formation of Myosin II filaments under the regulation of cGMP (93). cGMP activates GbpC which results in phosphorylation of Myosin light chain kinase A (94–97). sGC, suggested to be activated downstream of Ras (53), is the main producer of cGMP in response to cAMP and folate. However, sGC and GbpC were found in the screen with Gβγ and not with any other bait including Ras. In addition, according to our recent data, Gβγ interacts directly with sGC and GbpC, strongly suggesting that Gβγ and not Ras (53) is the major regulator of the cGMP pathway (data not shown). cGMP is a fast diffusing signalling molecule inducing myosin filaments, thereby acting as a global inhibitor for F-actin induced protrusions (98, 99).

**Conclusions**

The research work presented here shows that Mass Spectrometry is a promising approach to gain new insights into the molecular mechanisms underlying chemotaxis. The novel, non-canonical regulators of heterotrimeric G-protein cycle were identified along with few other proteins that might serve as connecting link between heterotrimeric G-proteins and Ras proteins. Importantly we found several hits that directly connect Gα-signaling to actin cytoskeleton, strengthening the role of Gα-proteins as local activators of signaling cascade leading to F-actin polymerization at the leading edge. Furthermore, evidence from MS data along with our recent unpublished findings suggest that Gβγ via cGMP/myosin is the global inhibitor, thereby, inhibiting pseudopod formation at the sides and at the back of the cell.
Thus, the combination of Dictyostelium as a model organism, along with affinity purification of bait proteins and MS based proteomic approach, has resulted in identification of a lot of crucial components. The next challenge is to determine the contribution of the different identified proteins in the signaling pathways.

**Materials and Methods**

**Protein purification**

All bait proteins were expressed from either pGEX4T3 or pGEX4T1 plasmid containing an N-terminal GST and thrombin or TEV cleavage side respectively (GE Healthcare) as described before (22, 23, 77). Proteins were isolated, like previously described, by GSH affinity, cleavage and size exclusion chromatography. The purified proteins were analyzed by SDS-PAGE, and the concentration was determined by Bradford’s method (Bio-Rad).

**Interaction screen**

3mg of recombinant GST-tagged purified proteins (each bait protein) was bound to the GSH column (Glutathione Sepharose 4 Fast flow, GE Healthcare) and circulated with lysate of 1X10^9 Dictyostelium discoideum cells overnight. The lysate was prepared by incubating cells on ice for 30 min in assay buffer (50mM Tris pH 7.5, 50mM NaCl, 5mM DTT, and 5mM MgCl₂) containing 1% TritonX-100 and 1mg/ml of crushed protease inhibitor tablets (Roche). The lysate was cleared by centrifuging at 12,000 u g at 4°C. The unbound proteins were removed by washing the column with 100ml of assay buffer containing 500mM NaCl. The bait proteins were eluted along with their interacting partners using 50ml elution buffer containing 20mM glutathione (pH 7.5) and concentrated using centrifugal concentrators (Milipore, Amicon).

**Protein identification by mass spectrometry**

Protein samples were concentrated and separated by 1D-SDS-PAGE. After Coomassie staining each lane was cut into 24 slices and subjected to in-gel digestion with trypsin (Trypsin Gold, Promega), prior reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide. Peptide mixtures were trapped on C18 reversed phase EASY-Column and separated on a 100 mm C18 reversed phase column (75 μm × 100 mm, 3-μm particle size, Thermo Scientific) using a linear gradient from 0% to 35% B (A = 0.1% formic acid; B = 100% (v/v) acetonitrile, 0.1% formic acid) over 70 min at a constant flow rate of 300 nL/min. Nanoflow LC-MS/MS was performed on an EASYII LC system (Thermo Scientific) coupled
to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) operating in positive mode. MS scans were acquired in the Orbitrap in the range from 350 to 1800 m/z, with a resolution of 60,000 (FWHM). The 7 most intense ions per scan were submitted to MS/MS fragmentation (35% Normalized Collision Energy™) and detected in the linear ion trap. Peak lists were obtained from raw data files using the Proteome Discoverer v 1.3 software. Mascot (version 2.1, MatrixScience) was used for searching against a sequence database obtained by combining the *E. coli* with the *Dictyostelium* proteome sequences. The peptide tolerance was set to 40 ppm and the fragment ion tolerance to 2.0 Da, using semi-trypsin as protease specificity and allowing for up to 2 missed cleavages. Oxidation of methionine residues, deamidation of asparagine and glutamine, and carboxamidomethylation of cysteines were specified as variable modifications. Peptide and protein identifications were further validated with the program Scaffold (Version Scaffold 3.2, Proteome Software Inc., Portland, OR). Protein identifications based on at least 2 unique peptides identified by MS/MS, each with a confidence of identification probability higher than 95%, were accepted.

**Data analysis**

The whole set of proteins identified in each condition was normalized to correct for low expression or low loading of the samples, using six house-keeping proteins, namely, DSC-C1, ATP5B, ABPA (or ACTNA), ABPC (or GELA), MDHB, GPDA (or G3P). Data were Z-transformed prior to clustering and protein selection as follows. The normalized counts were standardized for each sample by calculating the Standardized score (Zp) for each protein-bait combination according to

\[ Z_p = \frac{X_p - \mu}{\sigma} \]

*XP* is spectral count for protein *P*

*μ* is average count in the sample of the specific bait

*σ* is standard deviation of counts in the sample.

The strength of a connection between bait and a specific protein was determined using Zp score and counts. Proteins were regarded as specific for a certain condition when the following criteria were met.

- Protein should have a z-score > 0.05 in the condition of interest.
- Proteins should have a z-score < 0.05 in the Roco4 condition that is used as control.
- Protein should have an absolute count > 5 in the condition of interest
Gene annotations for the SwissProt accession numbers were obtained from dictybase (http://dictybase.org). Statistical package R (www.r-project.org) was used for data analysis and graph drawing.

References