Guanylyl Cyclase Protein and cGMP Product Independently Control Front and Back of Chemotaxing Dictyostelium Cells

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Chemotaxis of amoeboid cells is driven by actin filaments in leading pseudopodia and actin–myosin filaments in the back and at the side of the cell to suppress pseudopodia. In Dictyostelium, cGMP plays an important role during chemotaxis and is produced predominantly by a soluble guanylyl cyclase (sGC). The sGC protein is enriched in extending pseudopodia at the leading edge of the cell during chemotaxis. We show here that the sGC protein and the cGMP product have different functions during chemotaxis, using two mutants that lose either catalytic activity (sGCΔcat) or localization to the leading edge (sGCΔN). Cells expressing sGCΔN exhibit excellent cGMP formation and myosin localization in the back of the cell, but they exhibit poor orientation at the leading edge. Cells expressing the catalytically dead sGCΔcat mutant show poor myosin localization at the back, but excellent localization of the sGC protein at the leading edge, where it enhances the probability that a new pseudopod is made in proximity to previous pseudopodia, resulting in a decrease of the degree of turning. Thus cGMP suppresses pseudopod formation in the back of the cell, whereas the sGC protein refines pseudopod formation at the leading edge.

INTRODUCTION

Chemotaxis is a vital process in a variety of organisms, ranging from bacteria to vertebrates. Prokaryotes use chemotaxis to move toward high nutrient concentrations or away from unfavorable conditions, whereas in eukaryotes chemotaxis is also involved in embryogenesis, wound healing, and the immune response. Chemotaxis is achieved by coupling gradient sensing to basic cell movement. Prokaryotes are too small to sense spatial gradients and therefore rely on temporal changes of the chemoattractant concentration to achieve chemotaxis. They do this by adjusting their tumbling frequency in response to temporal changes of the chemoattractant concentration (Szmurman and Ordal, 2004; Wadhams and Armitage, 2004). Eukaryotic cells are typically large enough to be able to measure a spatial gradient. The difference in receptor occupation between each side of the cell leads to an internal polarization. A pseudopod is extended at the side with the highest receptor occupation and at the same time, pseudopod formation at all other sides is repressed, resulting in directional cell migration (Devreotes and Janetopoulos, 2003; Postma et al., 2004).

Dictyostelium is a eukaryotic organism that is widely used to study chemotaxis (Williams and Harwood, 2003; Parent, 2004; Affolter and Weijer, 2005). Starved Dictyostelium cells periodically secrete cAMP. Through relay of the cAMP signal by neighboring cells, concentric cAMP waves are generated. Starved Dictyostelium cells are chemotactically sensitive to cAMP and by movement in the direction of the origin of the cAMP waves, the cells are able to aggregate into groups of up to 100,000 cells. The chemotactic response of Dictyostelium is optimized for the dynamic cAMP waves that coordinate both aggregation and multicellular development. Cells show a much stronger chemotactic response to a cAMP wave, where the mean concentration increases over time, than to a static spatial gradient. Dictyostelium uses both spatial gradient sensing and the “bacterial-like” temporal gradient sensing to respond to these dynamic chemoattractant gradients (Futrelle, 1982; Varnum-Finney et al., 1987; Iijima et al., 2002; Xu et al., 2005).

The signal transduction pathways that are coupled to spatial gradient sensing and temporal gradient sensing rely on signal molecules with differential physical properties. Molecules that store spatial information have low diffusion rates to prevent the information from simply diffusing away. Because lipid molecules have very low diffusion constants (Almeida and Vaz, 1995), the phosphatidyl inositol phosphates that are formed during chemotaxis are excellent molecules to store spatial information (Funamoto et al., 2001; Wang et al., 2002). In contrast, molecules that carry temporal information benefit from high diffusion rates, which allow a rapid propagation of the signal throughout the cell. Small soluble molecules have high diffusion rates and are therefore most suitable as signal molecules for temporal signal transduction. One of the second messengers used during Dictyostelium chemotaxis is cGMP, which mediates the formation of myosin filaments in response to a cAMP stimulus (Mato and Malchow, 1978; Liu and Newell, 1988; Van Haastert and Kuwayama, 1997). Being a small molecule, cGMP has a very high diffusion constant of ~300 μm²/min (Allbritton et al., 1992; Chen et al., 1999). It can be calculated that the average...
dispersion length of a cGMP molecule is 50 μm, which is about 5 times the size of a Dictostelium cell (Postma and Van Haastert, 2001). This makes cGMP unsuitable to store spatial information but an excellent candidate to transduce temporal information. The predominant source of cGMP during development is soluble guanylyl cyclase (sGC) (Roelofs et al., 2001a; Roelofs and Van Haastert, 2002). With a molecular mass of 315 kDa, sGC is an unusually large protein. The protein is enriched in pseudopodia and localizes to the leading edge of the cell in a chemoattractant gradient (Veltman et al., 2005), where it exhibits very slow diffusion (D = 6 μm²/min; Ruchira Engel, unpublished data). With the observed localization and its slow diffusion, sGC has the potential to store spatial information. Thus, the sGC protein has opposite properties from its cGMP product, and the combination of sGC localization and transient cGMP formation allows the processing of both spatial and temporal information during chemotaxis. In this article, we explore the role of the localization of the sGC protein and formation of cGMP product during chemotaxis by generating two mutants of the sGC protein that uncouple localization of the protein and catalytic activity. Deletion of the N-terminal segment of sGC yields a protein that retains guanylyl cyclase activity but loses its localization to the leading edge. Cells expressing this protein exhibit excellent chemotaxis during the rising flank of a cAMP wave, but they lose their orientation in a stable spatial gradient. The second mutant contains a point mutation of a catalytic amino acid, yielding a protein that has no guanylyl cyclase activity but still localizes to the leading edge. Cells expressing this catalytically dead mutant show poor chemotaxis in a spatiotemporal gradient, but they improve chemotaxis in stable spatial gradients to the level of wild-type cells. These data suggest that localization of the sGC protein in pseudopodia and the leading edge retains spatial information and stabilizes the leading edge, whereas transient cGMP formation induces myosin filament formation and suppresses pseudopod formation in the back and at the sides of the cell.

MATERIALS AND METHODS

Strains and Culture Conditions

All cell strains were grown in HG5 medium (143 g/l oxoid peptone, 7.15 g/l bacto yeast extract, 1.36 g/l Na₂HPO₄·12H₂O, 0.49 g/l KH₂PO₄, and 10.0 g/l glucose). When grown with selection, HG5 medium was supplemented with 10 μg/ml G418 or 50 μg/ml hygromycin. Previous to cGMP activity assays and confocal microscopy, cells were starved for 5 h by shaking in 17 mM glucose. When grown with selection, HG5 medium was supplemented with 10 μg/ml G418 or 50 μg/ml hygromycin. Previous to cGMP activity assays and confocal microscopy, cells were starved for 5 h by shaking in 17 mM glucose.

Cloning of the Expression Constructs

Plasmid pBK-CMV/sGC (Veltman et al., 2005) that contains the entire open reading frame (ORF) of sgc (GenBank AF361947) was used as starting material to construct the various mutants. The ORF of sgc is preceded by a PstI and BamH1 site. The stop codon is immediately followed by a BamH1 site. In the downstream, internal atg reading frame (ORF) of sgc was excised from pBK-CMV/sGC by using PstI and KpnI. KpnI recognizes the internal 5′-ggaaatacatcaccaatgct-3′ together with a 3′-ggaaatacatcaccaatgtc-5′ cassette that was obtained by digesting plasmid pHygTm(plus) (a kind gift of Jeff Williams, Developmental Biology, University of Dundee, Dundee, United Kingdom) with BstXI and HindIII. The BstXI site of the fragment was made blunt by a Klenow fill-in. A linear knockout fragment was obtained by PCR, using forward primer accattgcaagagtac and reverse primer tga- catcttacacc: on plasmid pMD1000, gco-null cells (Roelofs et al., 2001b) were transolated with 5 μg of the purified PCR product. Cells were selected with 35 μg/ml hygromycin, and clonal transfectants were isolated.

In Vivo cGMP Assays

In Vitro Guanylyl Cyclase Activity Assays

Starved cells were washed and resuspended in 10 mM Tris, pH 8.0, to a density of 2 × 10⁶ cells/ml. All subsequent steps were performed at 4°C. One volume of cell suspension was mixed with 1 volume of lysis buffer yielding 15 mM Tris, 250 mM sucrose, and 3 mM EGTA at pH 7.4, and lysed through a 4-μm Nucleopore filter. Guanylyl cyclase assays were performed at 22°C with 50 μl of cell lysate in a total volume of 100 μl containing 15 mM Tris, pH 8.0, 250 mM sucrose, 0.5 mM GTP, 10 mM 1,4-dithiothreitol, 1.5 mM EGTA, and 2.5 mM MnCl₂ (final concentrations). Reactions were terminated after 20, 40, and 60 s by addition of 40 μl of assay mixture to an equal volume of 5.5% perchloric acid. Samples were neutralized by adding 20 μl of 50% saturated KHC0₃ and incubated for 5 min at room temperature to allow the CO₂ to escape. Samples were then centrifuged 5 min at 30,000 × g at 4°C. The cGMP level in each supernatant was determined using the Biotrak [³H]cGMP assay kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) according to manufacturer’s instructions.

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direction of the gradient and its traveled distance), yielding a series of chemotaxis indices for each cell in the movie. To determine the degree of turning, the displacement of the cells during 1 min was calculated as a vector. The degree of turning is defined as the angle between subsequent vectors. At least four movies were analyzed for each cell strain, and ~20 individual cells were analyzed per movie. The data shown are the average and SE of the mean, with n representing the number of cells analyzed.

Fluorescence of the expressed GFP and monomeric Kusabira-Orange (mKO) fusion constructs was visualized using a Zeiss LSM510 (Carl Zeiss, Jena, Germany) confocal fluorescence microscope fitted with a Plan-Neofluar 40× 1.3 numerical aperture oil immersion objective. The collected fluorescence data were quantified on a computer using the MatLab program (Mathworks, Natick, MA).

RESULTS

Localization and Activity of Two Mutant sGC Enzymes

Two mutants of the Dictyostelium sGC were constructed (Figure 1A). In the first mutant, termed sGCAN, the N-terminal 877 amino acids were deleted. In the second construct, termed sGCacat, a point mutation was introduced in the catalytic site. The aspartate at position 1106, which coordinates the magnesium ion during the cyclization reaction, was substituted for an alanine residue, rendering the enzyme inactive (Olson et al., 1998). Wild-type sGC and mutant sGC constructs were fused at the C terminus to GFP and expressed in gc-null cells. The Dictyostelium genome encodes two guanylyl cyclases, GCA and sGC. Both cyclases have been knocked out in the gc-null cell strain, and as a consequence, these cells are no longer able to synthesize cGMP. It has previously been shown that full-length sGC-GFP localizes to the leading edge of the cell during chemotaxis; the fluorescence intensity at the leading edge is about twofold higher than the fluorescence intensity in the cytosol (Veltman et al., 2005). The catalytically inactive mutant shows a similar, even somewhat more pronounced, anterior localization as wild-type sGC (Figure 1B and Supplemental Movie). In contrast, sGCAN remains entirely cytosolic, suggesting that the N-terminal region targets the enzyme to the leading edge. A GFP-fusion protein, termed N-sGC', that consists of only the N-terminal region of sGC showed a similar localization as the full-length sGC. This indicates that the N-terminal region is not only required but also sufficient for anterior localization. In an attempt to identify a minimal fragment that is sufficient for the observed localization, we expressed another five GFP-fusion constructs that spanned amino acids 1-305, 306-680, 681-1019, 1-680, and 306-1019, respectively. However, none of these GFP-fusion proteins showed significant anterior localization (our unpublished data), indicating that the entire N-terminal region is required.

Next, the guanylyl cyclase activity of the sGC mutant cell strains in vitro and the chemoattractant-stimulated cGMP response in vivo were measured, which are both absent in the parent gc-null cells (Figure 1C). A lysate of gc-null cells expressing the wild-type protein (sGC') showed a guanylyl cyclase activity of ~100 pmol cGMP/min/10^5 cells (Figure 1C). The rate of cGMP synthesis of lysates of gc-null cells expressing N-terminally truncated sGC (sGCAN') is almost identical to that of sGC' cells. The in vivo cAMP-stimulated cGMP accumulation of starved sGCAN' cells is comparable with that of sGC' cells, indicating that the N-terminal region is not required for catalytic activity (Figure 1D). gc-null cells expressing catalytically inactive sGC (sGCacat'), as expected, did not yield any detectable levels of cGMP in both the in vitro guanylyl cyclase assays and in vivo response assays.

The opposite characteristics of sGCAN and sGCacat make up for a valuable set of mutants: sGCAN' cells show a proper temporal cGMP response to an extracellular cAMP concentration increase, but they show no spatial localization of the sGC protein in a cAMP gradient. In contrast, sGCacat' cells do not synthesize any cGMP in response to a CAMP stimulus, but the protein still localizes to the leading edge of the cell in a gradient. Therefore, with these mutants we have effectively uncoupled the cGMP response and localization of the sGC protein.

Chemotaxis Efficiency in Dynamic and Static cAMP Gradients

Dictyostelium chemotaxis is optimized to respond to dynamic cAMP waves. Many signal transduction pathways that are involved in chemotaxis, including the cAMP pathway, are activated by an increase of the cAMP concentration and adapt when the concentration remains constant (Dinauer et al., 1980; Van Haastert and Van der Heijden, 1983). Therefore, we have devised a chemotaxis chamber that mimics the spatiotemporal gradient of the rising flank of the natural CAMP wave (see Materials and Methods and Figure 2). Cells were placed under the glass bridge of this modified Zigmond chemotaxis chamber. The cells are exposed to a spatial and temporal gradient between 1 and 10 min after...
Cells expressing the wild-type protein (sGC\textsubscript{oe}) exhibit a chemotaxis index of each 30-s step is plotted in Figure 3. The coordinates of the cells were determined during 35 min at a distance of 700 m, which equals approximately the length of a Dictyostelium cell.

The coordinates of the cells were determined during 35 min at a 30-s interval immediately after application of the cAMP source. From these data, the chemotaxis index gradually increases and reaches the value of gc-null cells at the end of the experiment (Table 1). Cells expressing sGC\textsubscript{cat}, the catalytically inactive sGC protein that localizes to the leading edge, exhibit very poor chemotactic response to the initial spatiotemporal gradient, with a chemotaxis index that is even lower than that of gc-null cells. However, the chemotaxis index gradually increases and reaches the value of cells expressing the wild-type protein at the end of the experiment (Table 1).

The results of Figure 3 show that the mutants exhibit a very different response to the spatiotemporal gradient compared with the static spatial gradient. In a spatiotemporal gradient, cells with a cGMP-plus phenotype (sGC\textsubscript{oe} and sGCAN\textsubscript{m}) have a much better chemotaxis response than cGMP-null cell strains (gc-null and sGC\textsubscript{cat}m), and localization of the sGC protein has only minor effects. In contrast, in a stable spatial gradient, cells with anterior localization of sGC (sGC\textsubscript{oe} and sGC\textsubscript{cat}m) have a much better chemotaxis response than sGC\textsubscript{oe} cells, i.e., gc-null cells expressing the wild-type sGC protein, are similar to that of the response of wild-type AX3 cells (our unpublished data). The chemotaxis index of gc-null cells is significantly lower than that of cells expressing the sGC protein. The cells lack the sharp increase in response to the spatiotemporal gradient and reach a maximum chemotaxis index of only 0.43. Cells expressing sGC\textsubscript{AN}m, the sGC mutant that produces cGMP but does not localize to the leading edge, exhibit a very good chemotaxis response during the initial spatiotemporal gradient, being essentially identical to that of cells expressing the wild-type protein. However, in the static gradient the chemotaxis efficiency slowly declines until it reaches the value of gc-null cells at the end of the experiment (Table 1). Cells expressing sGC\textsubscript{cat}, the catalytically inactive sGC protein that localizes to the leading edge, show very poor chemotactic response to the initial spatiotemporal gradient, with a chemotaxis index that is even lower than that of gc-null cells. However, the chemotaxis index gradually increases and reaches the value of cells expressing the wild-type protein at the end of the experiment (Table 1).
Table 1. Chemotaxis efficiency and degree of turning of mutant sGC cell strains

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Spatiotemporal gradient</th>
<th>Early spatial gradient</th>
<th>Late spatial gradient</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Chemotaxis index (°/min)</td>
<td>Directional change (°/min)</td>
<td>Chemotaxis index (°/min)</td>
</tr>
<tr>
<td>gc-null</td>
<td>0.40 ± 0.04</td>
<td>35.7 ± 1.5</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>sGC</td>
<td>0.58 ± 0.03**</td>
<td>28.6 ± 1.5**</td>
<td>0.60 ± 0.04**</td>
</tr>
<tr>
<td>sGCΔN</td>
<td>0.56 ± 0.03**</td>
<td>31.5 ± 1.2**</td>
<td>0.57 ± 0.04**</td>
</tr>
<tr>
<td>sGCΔcat</td>
<td>0.35 ± 0.04</td>
<td>35.0 ± 1.7</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>sGCΔNΔcat</td>
<td>0.34 ± 0.05</td>
<td>34.7 ± 3.6</td>
<td>0.40 ± 0.06</td>
</tr>
<tr>
<td>sGCΔcatΔN</td>
<td>0.41 ± 0.04</td>
<td>34.8 ± 3.0</td>
<td>0.29 ± 0.07</td>
</tr>
</tbody>
</table>

The chemotaxis efficiency and degree of turning of mutant sGC cell strains in the modified Zigmond chemotaxis chamber in response to the spatiotemporal gradient (average of a 5-min window at t = 15 min) and the late spatial gradient (average of a 5-min window at t = 30 min) are shown. The degree of turning is defined as follows. For each 1-min step, the displacement of the cells was calculated as a vector. The degree of turning is the angle between subsequent vectors. The data shown are the average and SE of the mean, with n representing the number of cells analyzed.

* indicates that the value is significantly different from the value of gc-null cells at *p < 0.05 or **p < 0.01; data without asterisk(s) are not significantly different at p > 0.05.

response than cells without sGC localization (gc-null and sGCΔNcat), and cGMP is of less importance. To further investigate the function of cGMP, image sequences of a representative cGMP-plus cell (sGCΔN) and cGMP-null cell (gc-null) during the spatiotemporal gradient are presented in Figure 4A. In the absence of a chemotactic gradient, cells from both strains extend multiple pseudopodia in random directions. As soon as the cAMP-wave arrives at the cell (top frame), both cells extend a pseudopod in the proper direction. This indicates that directional sensing is largely intact in cGMP-null cells. However, only cells with a proper cGMP response retract all remaining pseudopodia, resulting in an elongated cell shape and coordinated movement in the direction of the gradient, whereas cGMP-null cells continue to extend pseudopodia in the back of the cell, thereby compromising their chemotaxis efficiency. The cell tracks in Figure 4B show that the smooth path with relatively few lateral pseudopodia in cGMP-plus cells is maintained throughout the course of the experiment.

Cells expressing the catalytically inactive sGCΔcat lack the rapid reorientation toward the cAMP source during the initial spatiotemporal gradient. However, the cells eventually acquire a chemotaxis index that is as high as cells expressing wild-type sGC. To investigate the mechanism by which sGCΔcat attains this high chemotaxis index, we have determined many characteristics of sGCΔcat cells in late spatial gradients, such as speed, directional change, and persistence of movement. None of these were significantly different between gc-null and sGCΔcat cells, except for the directional change, which is 38.4°/min in gc-null cells and 30.5°/min in sGCΔcat cells (Table 1). Interestingly, in the early spatial gradient, 15 min after initiation of gradient formation, sGCΔcat cells still show poor chemotaxis but already have a significantly lower degree of turning than gc-null cells (Table 1). These results indicate that sGCΔcat cells still make lateral pseudopodia, but these do not become dominant. Instead, the leading edge makes less turns, thereby improving its orientation in the chemotactic gradient and allowing the cells to eventually acquire a chemotaxis index that is as high as cells expressing wild-type sGC.

Cells expressing the catalytically active but cytosolic sGCΔN have the opposite behavior. These cells show the rapid reorientation toward the cAMP source during the initial spatiotemporal gradient, but exhibits a high directional change in the late spatial gradient. The smooth cell tracks of sGCΔN throughout the experiment (Figure 4B) suggests that sGCΔN cells maintain the suppression of lateral pseudopodia in a stable spatial gradient but that the leading edge slowly loses orientation due to the high degree of turning.

To identify the parts of the sGC protein that are essential for the improved anterior orientation by the sGCΔcat protein, we expressed N-terminal and C-terminal deletions of sGCΔcat in gc-null cells. As expected, deletion of the N terminus (sGCΔNΔcat) yields a protein with homogenous cytoplasmic localization (our unpublished data). The chemotaxis data presented in Table 1 shows that these sGCΔNΔcat cells exhibit a similar chemotaxis response as gc-null cells. The C-terminal region of sGC is ~1000 amino acids long and contains a well conserved AAA+ ATPase domain. Deletion of this C-terminal region from the catalytically inactive sGC (sGCΔNΔcat) yields a protein that still localizes to pseudopodia and the leading edge (our unpublished data), but no longer supports chemotaxis (Table 1). This suggests that the contribution of the sGC protein to chemotaxis in the spatial gradient requires both the N-terminal region which targets the protein to the pseudopodia and the C-terminal region containing the AAA+ ATPase domain.

**Effects of sGC and cGMP on Myosin Distribution**

In wild-type chemotaxing Dictyostelium cells, high levels of filamentous myosin are found in retracting pseudopodia and the posterior cell cortex (Clow and McNally, 1999). In vitro assays have shown that the cAMP-stimulated incorporation of myosin filaments into the Triton-insoluble cytoskeleton is absent in gc-null cells (Liu et al., 1993; Bosgraaf et al., 2002). To investigate the effects of the sGC protein and the cGMP product on myosin incorporation into the cytoskeleton, we coexpressed myosin-GFP in the various mutant sGC cell strains. Cells were placed under the bridge of the modified Zigmond chemotaxis chamber and allowed to migrate toward the cAMP. Cells expressing full-length sGC quickly become elongated in the cAMP gradient. In these elongated cells, a large amount of myosin is found along the posterior cell cortex (Figure 5). In contrast, in gc-null cells, most of the myosin remains cytosolic. A small myosin fraction is found in retracting pseudopodia, but the cells lack the pronounced incorporation of myosin along the posterior cell cortex. In

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According to the sequenced evidence, the cells remain mostly nonpolar. Expression of the mutant sGC\textit{H9004N} protein, which no longer localizes to the leading edge but still synthesizes cGMP, rescues both the elongated cell shape and the myosin incorporation in the posterior cell cortex. In contrast, cells expressing the catalytically inactive sGC\textit{H9004cat} show a similar phenotype as gc-null cells; myosin remains mostly cytosolic and cells do not adopt an elongated cell shape, indicating that the regulation of myosin during chemotaxis is dependent only on cGMP and not on the localization of the sGC protein. The shape of sGC\textit{H9004cat} cells and the distribution of myosin-GFP as shown in Figure 5 do not change much during the chemotaxis assay. Thus, at 30 min after stimulation, when the sGC\textit{H9004cat} cells have a high chemotaxis index, myosin does not become enriched in the back of the cell, cells do not adopt an elongated cell shape, and cells still make pseudopodia at the back of the cell.

**Distribution of Filamentous Actin and sGC**

Filamentous actin is the major constituent of the cytoskeleton in extending pseudopodia. To investigate the relation between F-actin distribution and the localization of sGC during chemotaxis, the filamentous actin binding protein LimE\textit{coil} (Schneider \textit{et al.}, 2003) was C-terminally fused to mKO (Karasawa \textit{et al.}, 2004) and used as an F-actin probe in the various cell strains expressing the GFP-tagged sGC mutants. The cells were placed in a cAMP gradient and visualized by confocal microscopy. Most F-actin in the chemotaxing cells was detected in protruding pseudopodia at the leading edge. Interestingly, all sGC mutant proteins with an intact N-terminal domain (sGC-GFP, sGC\textit{H9004cat-GFP [shown in Figure 6A] and N-sGC\textit{1-1019}}) were found to colocalize to a large extent with LimE\textit{coil-mKO}, indicating that sGC may interact with F-actin at the leading edge of the cell. To investigate whether the anterior localization of sGC is dependent on the presence of F-actin, we depolymerized all actin filaments by treating the cells with 5\textit{\mu}M latrunculin A, a molecular compound that traps actin in its monomeric state. Treated cells become spherical and, as expected, the distribution of LimE\textit{coil-mKO} is completely homogenous in these cells (Figure 6B). At the same time, distribution of the sGC mutant proteins in treated cells also becomes completely homogenous. It has previously been found that global stimulation of cells with cAMP greatly increases the amount of sGC in the cell cortex (Veltman \textit{et al.}, 2005). However, global stimulation of latrunculin A-treated cells no longer induces such a transient translocation of sGC to the cell cortex (Figure 6B). At the same time, distribution of the sGC mutant proteins in treated cells also becomes completely homogenous. It has previously been found that global stimulation of cells with cAMP greatly increases the amount of sGC in the cell cortex (Veltman \textit{et al.}, 2005). However, global stimulation of latrunculin A-treated cells no longer induces such a transient translocation of sGC to the cell cortex (Figure 6B). In addition, local stimulation by placing of a pipette with cAMP next to a latrunculin A-treated cell also no longer elicits a redistribution of sGC (Figure 6C). These results indicate that the localization of the sGC protein to the leading edge is dependent on the interaction of sGC with filamentous actin. In combination with the obtained chemotaxis data of the sGC\textit{H9004cat} mutant, this suggests that this interaction with the actin cytoskeleton is essential for the improved orientation that is observed in a stable cAMP gradient.
Chemotaxis of amoeboid cells is driven by actin filaments in leading pseudopodia and actin–myosin filaments in the back and at the side of the cell to suppress pseudopodia. Several signal transduction components are involved in regulating the actin–myosin cytoskeleton during chemotaxis, including phosphatidylinositol 3-kinase, PAK, WASP, and Ras (Tuxworth *et al*., 1997; Chung and Firtel, 1999; Lim *et al*., 2005; Myers *et al*., 2005). Mutants with deletions of the encoding genes suggest that none of these signaling pathways is essential but that each contributes to chemotaxis to a different extent. In *Dictyostelium*, cGMP plays an important role during chemotaxis and is produced predominantly by a sGC that is homologous to mammalian soluble adenylyl cyclase. The sGC protein is enriched in extending pseudopodia and activated during chemotaxis. We show here that the sGC protein and the cGMP product have different functions during chemotaxis. Together, they are responsible for about one-third of the chemotactic response as indicated by the chemotaxis index of gc-null cells of 0.43 compared with 0.64 of gc-null cells expressing the wild-type sGC protein, or wild-type AX3 cells. The chemotaxis index of gc-null cells is similar to the chemotaxis defects observed in PI3K-null cells (Funamoto *et al*., 2001; Loovers *et al*., 2006) but much larger than the minimal defects seen in PLC-null (Drayer *et al*., 1994) and adenylyl cyclase-null cells (Pitt *et al*., 1992; Stepanovic *et al*., 2005). In *Dictyostelium*, cGMP is involved in the regulation of myosin. Stimulation of wild-type cells with cAMP leads to a transient incorporation of myosin filaments in the cell cortex (Liu and Newell, 1994). Mutant cell strains with disrupted cGMP signaling show a strong reduction of this response (Bosgraaf *et al*., 2002). In a cAMP gradient, myosin is mostly found in the back of the cell, where it increases the cortical tension and suppresses pseudopod extension (Moores *et al*., 1996). We find that cGMP-null cells that are placed in a dynamic cAMP gradient incorporate almost no myosin in the posterior cell cortex during chemotaxis. Accordingly, mutants with disrupted cGMP signaling frequently extend pseudopodia at the back and sides of the cell, whereas cell strains with an elevated cGMP response are still intact in cells expressing the sGCΔN mutant, which shows a homogeneously cytosolic distribution but has a normal catalytic activity. This indicates that the cGMP-dependent myosin response is independent of the site where cGMP is synthetized.

We observe that the sGC protein associates with actin filaments in pseudopodia. The binding to filamentous actin is not essential for activation of sGC, because cells expressing the sGCΔN mutant that no longer associates to actin filaments in the leading edge still show a proper cGMP response to a cAMP stimulus. The role of the association of sGC to the actin cytoskeleton was investigated using a mutant with a point mutation in the catalytic site of the enzyme. The mutation renders the enzyme inactive and thereby...
blocks the ability of sGC to activate the cGMP signaling pathway. This catalytically inactive sGC\textsubscript{cat} protein is still present in pseudopodia at the leading edge. Expression of sGC\textsubscript{cat} in gc-null cells significantly enhances the chemotactic response in a static spatial chemoattractant gradient. This indicates that the sGC protein harbors additional functionality aside from cGMP synthesis. Expression of sGC\textsubscript{cat} leads to a decrease of the degree of turning of the cell from \(\sim 38\) to \(30^\circ/\text{min}\). Cells expressing wild-type sGC also show the low degree of turning, but cells expressing the cytosolic sGC\textsubscript{N} protein do not. We propose that filamentous actin-associated sGC modulates pseudopod formation, such that a new pseudopod is formed preferentially at a site close to an sGC-rich, old pseudopod. On disruption of actin filaments with lantrunculin A, sGC becomes completely cytosolic. Local stimulation of these lantrunculin A-treated cells with a pipette filled with cAMP has been shown to induce phosphatidyl inositol-(3,4,5)-trisphosphate formation at the side of the cell close to the pipette (Parent et al., 1998). We did not observe any translocation of sGC in such stimulated lantrunculin A-treated cells, indicating that sGC localization is not a primary response to the cAMP gradient, but it is dependent on the formation of actin filaments at the side of highest chemoattractant concentration. In a spatial gradient the sGC\textsubscript{cat} cells exhibit a reduced degree of turning before the chemotaxis index increases compared with gc-null cells. This suggests that the improvement of chemotaxis by sGC\textsubscript{cat} is the consequence rather than the cause of the reduced degree of turning. Collectively, these data suggest that the reduced degree of turning by the sGC protein creates a more dominant leading edge, which allows a more efficient chemotactic response of sGC\textsubscript{cat} cells compared with cells lacking anteriorly localized sGC.

The additional function of the sGC protein can potentially be mapped to two different regions of the enzyme. The protein consists of 2843 amino acids. The central cytosolic domain of \(\sim 800\) amino acids long is flanked on both sides by two regions of \(\sim 1000\) amino acids each, with a hitherto unknown function. The N-terminal region has a low complexity and shows no homology to any other sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). We have now identified that the N-terminal region is essential and sufficient for targeting sGC to the actin cytoskeleton in pseudopodia. It is most likely that the C-terminal domain is responsible for mediating the observed effects of sGC on chemotaxis, because expression of catalytically inactive sGC that lacks the C-terminal domain does not improve the chemotactic response of gc-null cells. The sequence of the C-terminal domain of sGC has been well conserved throughout evolution with orthologues in bacterial kinases and soluble adenyl cyclases. The domain contains a highly conserved P-loop ATPase motif that classifies sGC as a member of the AAA\textsuperscript{+} protein family (Leipe et al., 2004). Members of this family have been shown to use the energy released by the ATP\textsubscript{ase} activity to generate mechanical force (Neuwald et al., 1999). The C-terminal domain of sGC also contains signatures of the tetratricopeptide repeat motif, which is known to be involved in establishing protein–protein interactions (Sikorski et al., 1990; D’Andrea and Regan, 2003). The presence of these domains suggests that the increased anterior polarity induced by the sGC protein may be due to the active rearrangement of protein–protein interactions or the recruitment of additional proteins to the leading edge. This suggests that the sGC protein can be regarded as an actin-binding protein with AAA–ATP\textsubscript{ase} activity that modulates the local formation of pseudopodia.

Together, activation of sGC at the leading edge provides two very different but complementary sensory transduction pathways. Produced cGMP rapidly diffuses throughout the cell where it stimulates the incorporation of myosin in the cortex and inhibits pseudopod formation, whereas the sGC protein itself promotes the formation of a new pseudopod in the proximity of the old pseudopod. The sGC protein and cGMP product have opposite properties and functions. cGMP diffuses very fast by which it is expected to attain a spatially homogeneous concentration. Elevated cGMP levels are observed only after an increase of the chemoattractant concentration and adapt to constant concentrations (Van Haastert and Van der Heijden, 1983), indicating that cGMP senses the temporal component of the cAMP wave. In agreement with a function in temporal sensing, we observed that the sGC\textsubscript{N} mutant contributes to chemotaxis during the spatiotemporal part of the gradient but not during the stable spatial gradient. Alternatively, the sGC protein with its slow diffusion remains localized in pseudopodia in stable gradients, indicating that the sGC protein is involved in spatial sensing, which is strongly supported by the observation that sGC\textsubscript{cat} supports chemotaxis only in stable spatial gradients.

Several models have been proposed to explain how cells may read and respond to spatial gradients: local activation and global inhibition of pseudopod formation (Rappel et al., 2002; Ma et al., 2004), depletion of a cytosolic component to the leading edge (Postma and Van Haastert, 2001), or a bias of pseudopod formation by local activation at the front (Arrieumerlou and Meyer, 2005). Interestingly, the present observations on the function of cGMP and sGC provide essential elements for each of these models. Stimulation of pseudopod formation at the leading edge by sGC and inhibition of pseudopod formation in the back and at the side of the cell by cGMP represent the two components of the local activation and global inhibition model (Rappel et al., 2002; Ma et al., 2004). Recruitment of sGC to the leading edge also leads to a partial depletion of the limited amount of sGC from the cytosol. Models have been proposed that utilize the depletion of a cytosolic component to nonlinearily amplify the external chemoattractant gradient (Postma and Van Haastert, 2001). Finally, sGC reduces the degree of turning, supporting the local activation model. This model assumes that each receptor binding event within the leading edge triggers a local pseudopod extension and a small turn in the direction of migration. To achieve efficient chemotaxis, multiple steps are required where each subsequent step shows a slightly better orientation in the direction of the gradient. The relatively slow increase of chemotaxis efficiency of cells with anteriorly localized, catalytically inactive sGC in the spatial gradient is in excellent agreement with this model.

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**REFERENCES**


