Introduction

Chemotaxis is a pivotal response of many cells to spatial cues (Van Haastert and Devreotes, 2004; Affolter and Weijer, 2005; Wu, 2005). It plays important roles in diverse functions, such as finding nutrients in prokaryotes, forming multicellular structures in protozoa, and tracking bacterial infections in neutrophils (Baggiolini, 1998; Campbell and Butcher, 2000; Crone and Lee, 2002). Research on directional movement by external cues in eukaryotes is dominated by chemoattraction, which is the movement toward the chemical compound. Repellents play an important role in morphogenesis, especially during embryonic development (Yang et al., 2002; Schmitt et al., 2005). Cell movement during chick primitive streak formation is controlled by FGF8-mediated chemorepulsion of the cells away from the streak, followed by chemoattraction toward the FGF4 signal produced by the forming notochord (Yang et al., 2002). Axon guidance during spinal chord development away from the roof plate is regulated by multiple repellents, such as BMP (Butler and Dodd, 2003), and by the attractant netrin toward the floor plate (Kennedy et al., 2006).

The mechanism by which repellents work is not well known (Dormann and Weijer, 2006). We envision that a critical step of the signal transduction pathway for cell movement is stimulated by a chemoattractant and inhibited by a repellent. It is essential that this hypothetical step is somehow connected with cell polarity to obtain directional movement. Dictyostelium discoideum cells have been instrumental in resolving the mechanism by which cells sense and respond to chemoattractants. It has been shown that phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P_3), which is formed at the side of the cell closest to the source of chemoattractant, is a very strong inducer of pseudopod extensions (Parent et al., 1998; Hirsch et al., 2000; Servant et al., 2000; Funamoto et al., 2002; Iijima and Devreotes, 2002). D. discoideum cells are known to be repelled by unidentified compounds that are secreted by starving cells (Keating and Bonner, 1977; Kakebeeke et al., 1979), indicating that D. discoideum cells have a mechanism to process repellents. Previously, we have shown that several analogues of the attractant cAMP behave as a repellent (Van Haastert et al., 1984). The analogues mediate their effect through binding to the surface cAMP receptor cAR1 (Johnson et al., 1992), and they can be polar (3′deoxy, 3′amino-cAMP; 3′NH-cAMP) or lipophilic (8-para-chlorophenylthio-cAMP; 8CPT-cAMP). The analogues induce many signaling responses that are essentially identical to the responses induced by cAMP, including activation and adaptation of adenylyl and guanylyl cyclase (Peters et al., 1991; Bominaar and Van Haastert, 1993, 1994). We show that these analogues inhibit PLC, contrary to activation of PLC by cAMP. As a consequence, they induce dominant PI(3,4,5)P_3 signaling in the rear of the cell, by which cells move away from the repellent.
Results and discussion

*D. discoideum* cells were stimulated with a micropipette containing either the agonist cAMP or the commercially available antagonist 8CPT-cAMP. The cells moved toward the pipette with cAMP, but did not move effectively toward the pipette with 8CPT-cAMP, and actually moved away from the pipette (Fig. S1 and Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200611046/DC1). Experiments have been repeated with 3′NH-cAMP, yielding the same results as with 8CPT-cAMP (unpublished data). Fig. 1 A shows four frames from a movie in which cells were stimulated with two pipettes containing cAMP and 8CPT-cAMP, respectively (Video 3). In buffer, cells move in random directions (Fig. 1 A, 1 min), and cells move away from the pipette with 8CPT-cAMP (Fig. 1 A, 16 min). Upon application of the pipette with cAMP (cAMP and 8CPT-cAMP; Fig. 1 A, 26 min) cells moved in nearly random directions. However, upon withdrawal of the pipette containing 8CPT-cAMP, cells immediately moved toward the pipette with cAMP (Fig. 1 A, 38 min). The trajectories of the cells were analyzed. Data are presented as the chemotaxis index, which is the distance moved in the direction of the gradient (“upgradient”) divided by the total distance moved in 30-s intervals. Data from Video 3 are presented in Fig. 1 B, and the means and the SEMs for six independent experiments are presented in Fig. 1 C. Wild-type cells show an excellent chemotactic response toward cAMP, with a chemotaxis index of $0.81 \pm 0.05$. Cells are not attracted to the pipette containing 8CPT-cAMP, but instead exhibit a significant negative chemotaxis index of $-0.52 \pm 0.04$ ($P < 0.005$). The chemotaxis index of cells stimulated simultaneously with cAMP and 8CPT-cAMP is $-0.18 \pm 0.11$, indicating that 8CPT-cAMP antagonizes the positive chemotaxis toward cAMP and cAMP antagonizes the negative chemotaxis induced by 8CPT-cAMP. Finally, starting with stimulation by the two pipettes, upon withdrawal of the pipette with 8CPT-cAMP the chemotaxis index toward cAMP rapidly increases to $0.72 \pm 0.06$. The results demonstrate that 8CPT-cAMP is a repellent that can reversibly inhibit the chemotactic response to cAMP.

* D. discoideum* cells move using actin filaments in the front of the cell, which induce the formation of local pseudopodia, and actomyosin filaments in the rear of the cell, which inhibit pseudopod formation and retract the uropod. We coexpressed Myosin-RFP and the filamentous actin-binding protein LimE-GFP from a single plasmid. A pipette with cAMP induces the expected movement of the cells upgradient with LimE-GFP localized in the front and Myosin-RFP in the rear of the cell (Fig. 2 A and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200611046/DC1). Interestingly, the localization of LimE-GFP in the protruding front and Myosin-RFP in the retracting back is identical in cells stimulated with 8CPT-cAMP, except that the front is downgradient and cells move away from the pipette (Fig. 2 A and Video 5).
To investigate the mechanism by which 8CPT-cAMP induces negative chemotaxis, wild-type cells expressing the PI(3,4,5)P3 detector PHcracGFP were stimulated with cAMP and 8CPT-cAMP. Similar to previous investigations (Parent et al., 1998; Huang et al., 2003), a pipette with cAMP induces strong localization of PHcracGFP to the plasma membrane at the upgradient side of the cell. Pseudopodia are extended from PHcracGFP-containing areas and cells move upgradient toward the pipette (Fig. 2 B and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200611046/DC1). 8CPT-cAMP also induces strong localization of PHcracGFP at the plasma membrane, but with opposite polarity compared with cAMP, which is downgradient (Fig. 2 B and Video 7). Cells extend pseudopodia from these PHcracGFP-containing areas, and therefore move away from the pipette with 8CPT-cAMP. The size of the PHcracGFP patches induced by 8CPT-cAMP (9.0 ± 0.43 μm) is only slightly larger than the patches induced by cAMP (6.6 ± 0.17 μm), indicating that 8CPT-cAMP effectively reverses the PI(3,4,5)P3 polarity.

PI(3,4,5)P3 is formed by PI3-kinase (PI3K) and degraded by PTEN that, in cAMP gradients, are localized at the leading edge and the rear of the cell, respectively. In 8CPT-cAMP gradients, the localization of PI3K and PTEN is reversed compared with cAMP gradients (Fig. 2 B). To investigate the role of PI3K activity in polarity and chemotaxis reversal, we investigated the chemotactic activity of pi3k1/2−/−-null cells toward cAMP and 8CPT-cAMP. In pi3k1/2−/−-null cells, two PI3Ks are deleted that, together, mediate the vast majority of cAMP-stimulated PI(3,4,5)P3 production (Zhou et al., 1998; Funamoto et al., 2002; Huang et al., 2003). These experiments are possible because PI3K is not essential for chemotaxis, and directional sensing can be mediated by other pathways (Hirsch et al., 2000;...
Funamoto et al., 2002; Iijima and Devreotes, 2002; Huang et al., 2003; Postma et al., 2004; Loovers et al., 2006). Fig. 3 shows that $\pi_3k1/2$-null cells exhibit a good chemotactic response toward a pipette with cAMP (chemotaxis index is 0.80 ± 0.13). In contrast to the negative chemotaxis induced by 8CPT-cAMP in wild-type cells, $\pi_3k1/2$-null cells do not exhibit a significant negative or positive response to 8CPT-cAMP (chemotaxis index is 0.11 ± 0.12). More importantly, using two pipettes with cAMP and 8CPT-cAMP, respectively, $\pi_3k1/2$-null cells effectively move toward cAMP and are not inhibited by 8CPT-cAMP and cAMP. The results show that wild-type cells move away from 8CPT-cAMP, whereas $\pi_3k1/2$-null and plc-null are not repelled from 8CPT-cAMP. Moreover, chemotaxis toward cAMP is antagonized by 8CPT-cAMP in wild-type cells, but not in $\pi_3k1/2$null and plc-null cells. Videos 3, 8, and 9 are available at http://www.jcb.org/cgi/content/full/jcb.200611046/DC1.

The molecular mechanism by which cAMP mediates PI(3,4,5)P3 accumulation upgradient in $D. discoideum$ cells has been well described. PI3K is activated and enriched upgradient in the cell, whereas the PI(3,4,5)P3-degrading enzyme PTEN strongly localizes downgradient in the cell (Funamoto et al., 2002; Iijima and Devreotes, 2002). PTEN has been demonstrated to bind to phosphatidylinositol-3,4,5-trisphosphate (PI(4,5)P3), suggesting that PI(4,5)P3 is depleted upgradient in the cell (Iijima et al., 2004). This depletion of PI(4,5)P3 could be induced by several nonexclusive methods, such as the observed conversion of PI(4,5)P2 to PI(3,4,5)P3 upgradient by PI3K (Funamoto et al., 2002; Huang et al., 2003), but also by the conversion of PI(4,5)P2 to Ins(1,4,5)P3 and DAG by PLC, which is known to be activated by cAMP (Drayer and van Haastert, 1992; Bominaar et al., 1994). We propose a mechanism by which 8CPT-cAMP could revert the polarity of chemotactic sensing that is based on the observation that cAMP stimulates PLC, whereas 8CPT-cAMP inhibits this enzyme (Peters et al., 1991; Bominaar and Van Haastert, 1993; Bominaar and Van Haastert, 1994; supporting biochemical data are presented in Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200611046/DC1). Upgradient stimulation of PLC by cAMP will lead to local depletion of PI(4,5)P2, and thereby prevent PTEN binding, by which the upgradient PI(3,4,5)P3 accumulation is stabilized. In contrast, the upgradient inhibition of PLC by 8CPT-cAMP will lead to the local accumulation of PI(4,5)P2, thereby inducing PTEN binding and upgradient PI(3,4,5)P3 degradation (Fig. 4). This relatively simple model for polarity reversal predicts that 8CPT-cAMP does not induce polarity switching in plc-null cells. $D. discoideum$ cells contain a single plc gene encoding a PLCδ isofrom (Drayer and van Haastert, 1992), which, like PI3K, is instrumental but not essential for chemotaxis (Drayer et al., 1994). Expression of GFP-tagged reporter proteins in plc-null cells reveal, as predicted, cytosolic...
Polarization reversal by 8CPT-cAMP

In our model, a compound is a repellent because it binds to a receptor that is preferentially coupled to PLC via an inhibitory G protein, whereas it is an attractant when the receptor is preferentially coupled to PLC via an stimulatory G protein. The regulation of PLC by the stimulatory G2 and inhibitory G1 forms the basis for the polarity switch, and it allows the cell to respond to chemical gradient with repulsion or attraction. This polarity switch may be used by the cell during development. D. discoideum cells grow on bacteria. Cells starved for cAMP alone and, subsequently, move effectively toward an additional pipette with cAMP. This indicates that PLC is also essential for mediating the inhibitory effect of 8CPT-cAMP, as is PI3K. Finally, pten-null cells were investigated, showing that these cells are attracted toward cAMP, but are not repelled by 8CPT-cAMP (unpublished data).

A scheme for PI(3,4,5)P3-mediated chemotaxis reversal by 8CPT-cAMP consists of three parts (Fig. 4). The basis is a PLC/PI(4,5)P2 polarity switch. In D. discoideum, PLC is regulated by the activating Go2 and inhibitory Go1, which, in a gradient of attractant or repellent, will determine the polarity of the PI(4,5)P2 gradient. The attractant cAMP shows predominant activation of PLC, leading to lower PI(4,5)P2 levels upgradient, while the repellent 8CPT-cAMP inhibits PLC, leading to higher PI(4,5)P2 levels upgradient. The resulting gradients of PI(4,5)P2 and colocalized PTEN mediate opposite gradients of PI(3,4,5)P3, leading to the local polymerization of actin. The gradients of localized PTEN and PI3K are stabilized because PTEN accumulates at the site of its product PI(4,5)P2, whereas PI3K accumulates at sites of its effector, PI(3,4,5)P3-induced F-actin. This mutually exclusive interaction of PI3K and PTEN will result in symmetry breaking, by which small spatial differences in the underlying polarity gradient can be amplified to the observed strong PI(3,4,5)P3 gradient. Although PI3K and PLC are not essential for chemotaxis, the results clearly demonstrate that local formation of PI(3,4,5)P3 is a very strong inducer of pseudopod formation, such that the cells can even move downgradient, overriding any upgradient signaling that 8CPT-cAMP may induce.

In our model, a compound is a repellent because it binds to a receptor that is preferentially coupled to PLC via an inhibitory G protein, whereas it is an attractant when the receptor is coupled to a stimulatory G protein. The regulation of D. discoideum PLC by the stimulatory G2 and inhibitory G1 forms the basis for the polarity switch, and it allows the cell to respond to chemical gradient with repulsion or attraction. This polarity switch may be used by the cell during development. D. discoideum cells grow on bacteria. Cells starved for cAMP alone and, subsequently, move effectively toward an additional pipette with cAMP. This indicates that PLC is also essential for mediating the inhibitory effect of 8CPT-cAMP, as is PI3K. Finally, pten-null cells were investigated, showing that these cells are attracted toward cAMP, but are not repelled by 8CPT-cAMP (unpublished data).
Materials and methods

Plasmids and cells
The plasmid pWF38 (PHcracGFP) expressing the 700-bp N-terminal PH domain of CRAC fused to GFP (Parent et al., 1998), and plasmids expressing PI3K2-GFP (Funamoto et al., 2002; Iijima and Devreotes, 2002) and Pten-GFP (Iijima and Devreotes, 2002) were provided by P. Devreotes (Johns Hopkins University School of Medicine, Baltimore, MD). Plasmid 3395 expressing mRFPmars (Fischer et al., 2004) was provided by A. Muller-Taubenberger (Ludwig Maximilians University Munich, Munich, Germany). Plasmid pB2G-GF. myo expressing a GFP fusion with myosin heavy chain II (Levi et al., 2002) was a gift from T. Egelhoff [Case Western Reserve University, Cleveland, OH]. p3K1/2-null cells were provided by R. Firtel (University of California, San Diego, La Jolla, CA).

Plasmid IB15B expressing Lim-GFP and mRFP was constructed as follows. The neomycin resistance gene of MB74 was exchanged for the PFP hygromycin resistance gene that was preceded by an actin 15 promoter and terminated with a cab terminator. The DNA coding for the actin-binding domain of LimE (aa 1–145) was cloned behind an actin 15 promoter and 5 adenosines, which serve as the Kozak sequence. This was followed by a SpeI site (coding for Thr and Ser) and the complete open reading frame of GFP (S65T variant), followed by a stop codon and an actin 8 terminator. This yielded the plasmid MB74Hyg-Lim-GFP. The gene encoding the monomeric red fluorescent protein mRFPmars (Fischer et al., 2004) was amplified by PCR on plasmid DNA. The gene was preceded by a NgomIV site, an actin 15 promoter, and 5 adenosines, and was followed by a BamHI site (encoding Gly and Ser), the sequence encoding on 2–211 6 of myosin heavy chain, the myosin terminator from the vector pB2G-GFP, myo (Levi et al., 2002), and a NgomIV site. Finally, the gene encoding the mRFPmars-myosin fusion was released using the NgomIV site and cloned into the single NgomIV site of MB74Hyg-Lim-GFP.

Video 10 shows the localization of PHcracGFP, PTEN-GFP, and PI3K-GFP in mutant 3 NH-cAMP. 8CPT-cAMP has similar properties to 3 NH-cAMP. Video 5 shows the localization of PI3K-GFP (detecting PI[3,4,5]P3) at the leading edge of cells chemotaxing toward cAMP. Video 7 shows the localization of F-actin at the leading edge and myosin in the back of cells chemotaxing toward cAMP. Video 5 shows the localization of F-actin at the leading edge and myosin in the back of cells chemotaxing away from 8CPT-cAMP. Video 6 shows the localization of PHcracGFP (detecting PI[3,4,5]P3) at the leading edge of cells chemotaxing away from cAMP. Video 3 shows cell movement in gradients of 8CPT-cAMP. Video 9 shows chemotaxis of p3K1/2-null cells toward cAMP in the presence of 8CPT-cAMP. Video 8 shows chemotaxis of p3K1/2-null cells toward cAMP in the presence of 8CPT-cAMP. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200611046/DC1.

We thank B. Jastrow for a kind gift of 3 NH-cAMP. A. Bonairea for data on PI3K inhibition with 8CPT-cAMP. R. Firtel for p3K1/2-null cells. P. Devreotes for plasmids expressing PHcracGFP, PI3K2-GFP, and Pten-GFP. A. Muller-Taubenberger for plasmid expressing mRFPmars. T. Egelhoff for plasmid expressing a GFP fusion with myosin heavy chain II, and D. Bosgraaf for plasmid expressing Lim-GFP/MyoRFP. Plasmids and cells were obtained through the Dictyostelium Stock Center.

Submitted: 9 November 2006
Accepted: 18 April 2007

References


