PIP₃-dependent macropinocytosis is incompatible with chemotaxis

Douwe M. Veltman,¹ Michael G. Lemieux,² David A. Knecht,² and Robert H. Insall¹

¹Beatson Institute for Cancer Research, Glasgow G61 1BD, Scotland, UK
²Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269

Introduction

Eukaryotic cells sense chemoattractants using transmembrane receptors. When receptors detect attractants, they activate intracellular second messenger pathways that relay information to the molecules that drive cell movement. In Dictyostelium discoideum, one of the best organisms for studying chemotaxis (King and Insall, 2009; Cai and Devreotes, 2011), this simplification is even clearer: there are single genes for α and β subunits of the G proteins. Alpha subunits containing bound attractants cause G proteins to split from heterotrimers into active GTP-bound α subunits and βγ subunits, and these free subunits in turn activate further intracellular messengers. In cells such as neutrophils, with a vast range of receptors, this process simplifies the cell’s responses by limiting the number of active species that cause intracellular effects. In D. discoideum, one of the best organisms for studying chemotaxis (King and Insall, 2009; Cai and Devreotes, 2011), this simplification is even clearer: there are single genes for β and γ subunits (Wu et al., 1995; Zhang et al., 2001), and the βγ subunit is thought to mediate most responses. Thus, in theory all attractants (for example, cAMP and folate) should elicit fundamentally similar chemotaxis (Srinivasan et al., 2013). However, folate chemotaxis is often difficult to measure, for reasons explored in this paper.

Cells can be steered using many mechanisms. In shallow gradients, the migration process is fundamentally random, and attractants can bias multiple features of the normal motility cycle, for example the direction of new protrusions after pseudopod splitting (Bosgraaf and Van Haastert, 2009), and the probability of old pseudopods being retracted (Andrew and Insall, 2007). However, the second messengers that are used are still not understood. In one model, phosphatidylinositol 3,4,5-trisphosphate (PIP₃) is a “compass needle” (Bourne and Weiner, 2002); intracellular signal processing somehow resolves the ideal future direction of the cell, resulting in local generation of PIP₃ by the p110 phosphatidylinositol 3-kinase (PI 3-kinase) family. Discrete patches of PIP₃ appear at the leading edge of some cells during chemotaxis (Postma et al., 2004), which suggests that these represent the outcome of a nonlinear response, perhaps by discretizing spatial information to control protrusion (Cai and Devreotes, 2011). However, newer data have shown that PIP₃ is not essential for chemotaxis in D. discoideum.
Macropinocytosis also occurs in D. discoideum. In the wild, cells feed by phagocytosing bacteria. However, most laboratory work is done in axenic strains (“axenic” in this case means “able to grow in the absence of living prey”). These strains have been selected by growth in medium without bacteria, and are able to grow because of a constitutive high rate of macropinocytosis. D. discoideum macropinosomes are large structures (up to 5 μm) that efficiently take up liquid and soluble nutrients (Swanson, 2008). The mutations that allow axenic growth have been mapped to three separate loci, but their identity remains unknown (Clarke and Kayman, 1987). The use of axenic D. discoideum strains is so widespread that they are often, incorrectly, referred to as wild-type cells. In this work we show an unexpected conflict between chemotaxis and macropinocytosis that raises fundamental questions about the physiological role of PIP3.

Results and discussion

Axenic D. discoideum cells are defective in chemotaxis to folate

The main reason for studying chemotaxis in model organisms like D. discoideum is to find simple but generalizable results. It is therefore desirable to study multiple attractants to separate global from agonist-specific mechanisms. We have therefore studied chemotaxis toward folate. Compared with the well-studied cAMP system, folate uses different receptors and G protein α subunits (Srinivasan et al., 2013), which are expressed in growing cells that are not responsive to cAMP. However it has been difficult to measure folate chemotaxis with most assays (though under-agar and some micropipette assays succeed). To discover why, we exposed AX2 cells to folate gradients in a standard chemotaxis chamber (Muinonen-Martin et al., 2010). Under these conditions, starved cells chemotax efficiently toward cAMP. However, growing cells consistently failed to migrate up the folate gradient (Fig. 1 A). This was surprising, as folate is thought to be a potent attractant. To analyze the problem, we switched to using wild-type NC4, the parent strain of AX2, which does not have axenic mutations and must thus be grown on bacteria. In contrast to axenic cells, wild-type cells robustly migrated up the folate gradient (Fig. 1 B). AX2 cells grown on bacteria also show a little chemotaxis (Fig. 1 C), but still far less than NC4, which indicates that the loss of folate chemotaxis is caused by genetic differences between the wild type and the axenic AX2 strain.

Actin, pseudopod orientation, and PIP3

This defect in a strain that is widely used to study motility and chemotaxis is worrying, so we determined its cause. Chemoattractants steer cells by altering pseudopod behavior, so we measured pseudopod directions in axenic and nonaxenic cells. We also examined PIP3 because of its clear association with both macropinocytosis during axenic growth and chemotaxis. Cells were transfected with Lifeact-mRFP (Riedl et al., 2008) and PH-CRAC-GFP (Parent et al., 1998) to show actin pseudopods in red and PIP3 in green.

We first determined the fraction of F-actin–rich protrusions labeled with PIP3 during random migration (as observed by others, the PIP3-positive and -negative populations were essentially discrete, allowing an all-or-nothing score). In axenically cultivated AX2 cells, ~50% of random protrusions accumulate PIP3 at the edge (Fig. 2 A and B). In AX2 grown on bacteria, PIP3-containing pseudopods are only about half as frequent, though these cells migrate more efficiently.
**Figure 2.** **PIP3-labeled protrusions do not orient up folate gradients.** (A) AX2 and NC4 cells transfected with the F-actin reporter Lifeact-mRFP and the PIP3 reporter PH-CRAC-GFP were examined by confocal microscopy, and the presence or absence of a PIP3 patch in each cell was determined during random migration. Bar, 10 µm. (B) Numbers of pseudopods with and without PIP3 patches during random migration. Percentages are the means and standard deviations from three experiments. (C and D) Mean direction of protrusions during folate chemotaxis. (C) The displacements from the migration vectors of all pseudopods were measured, and the mean is shown in a circular plot. If all protrusions were extended in the same direction, a vector with length 1 would result. PIP3-containing pseudopods from NC4 were too rare to give a reliable direction. (D) Rose plots of the directions of all protrusions with and without PIP3. The shaded area indicates the 95% confidence interval of the mean angle. (E) Frequency and direction of pseudopods in C and D. (F) Rose plot of the direction of PIP3 patches that form macropinosomes during folate chemotaxis.
Expression of PIP₃ markers in wild-type cells

It has previously not been practical to express ectopic genes in wild-type *D. discoideum* because standard selectable markers and promoters work poorly in bacterially grown cells. We constructed new vectors to allow expression of markers for PIP₃ and F-actin in wild-type cells, and developed methods for transformation and selection using bacteria as food (see Materials and methods). We successfully cotransfected NC4 cells with Lifeact-mRFP and PH-CRAC-GFP to examine the distribution of F-actin and PIP₃. To our surprise, however, PIP₃-containing pseudopods were extremely rare in randomly moving wild-type cells (Fig. 2 B); 98% of pseudopods had no discernible PIP₃. Thus, in growing wild-type *D. discoideum*, unlike in axenic cells, PIP₃ appears not to be used at all in random migration, even though the cells migrate far more robustly.

**PIP₃ and folate chemotaxis**

We next examined PIP₃ during folate chemotaxis. Cells were exposed to a relatively steep folate gradient in a chemotaxis chamber, and the angle between each protrusion and the gradient was determined. As before, axenic AX2 cells did not show chemotaxis. The mean cosine of the angle between the protrusions and the chemoattractant gradient was close to zero, which indicates no alignment, regardless of whether PIP₃ was present. Also, protrusions with PIP₃ made a negligible contribution to net migration (Fig. 2, C, D, and E). Macropinosomes were not oriented by the folate gradient (Fig. 2 F).

As before, AX2 cells grown on bacteria chemotaxed weakly, and the proportion of pseudopods with PIP₃ patches decreased substantially (from 63% to 29%; Fig. 2 E). Furthermore, PIP₃-labeled protrusions did not orient up the gradient (Fig. 2 E). In contrast, the protrusions that showed no discernible PIP₃ oriented up the folate gradient with a mean cosine of 0.40. Thus, unexpectedly, absence of PIP₃ patches correlated with improved chemotaxis.

During chemotaxis, pseudopods in vegetative wild-type cells were still almost never labeled with PIP₃ (Fig. 2 E). In a folate gradient, the protrusions of wild-type cells showed high directionality (mean cosine 0.59) despite the lack of PIP₃ at the front (Fig. 2, C and D). Thus, in all conditions we observed, only PIP₃-negative pseudopods are directed by folate.

**PIP₃ and macropinocytosis in wild-type and axenic cells**

The negative correlation between the number of PIP₃ patches and the chemotaxis efficiency was unexpected, as PIP₃ signaling is thought to be fundamentally involved in directional sensing. However, PIP₃ is involved in several other physiological processes, in particular macropinocytosis (Dormann et al., 2004; Hoeller et al., 2013). We speculated that this could negatively impact chemotaxis efficiency, and we therefore examined macropinocytosis and its regulation by PIP₃ in more detail.

In wild-type cells, macropinocytosis could readily be visualized with the PIP₃ marker PH-CRAC-GFP (Fig. 3 A), despite the substantially lower rate of fluid uptake. The PIP₃ is strictly confined to the nascent vesicle; levels increase during invagination and reach a maximum as the vesicle closes. The entire process is very fast, with a mean time of 8 s between appearance of PIP₃ and budding of the vesicle (*n* = 27, SD = 4 s). Macropinosomes in wild-type cells are small and do not contribute to cell movement, and are readily distinguishable from the large, convex, and spiky F-actin pseudopods.

Unlike wild-type cells, axenic AX2 cells generate macropinosomes from preassembled PIP₃ patches. The PI 3-kinases responsible for this process have recently been analyzed in depth (Hoeller et al., 2013); the patches are presumably the structures organized by PIKs 1 and 2. The diameter of the patches is a...
substantial fraction of the size of the cell (Fig. 3 B). Progression of negative curvature to drive vesicle formation frequently stalls, causing patches to remain planar or slightly inwardly curved for prolonged periods of time. Moreover, PIP3 patches often disappear without any vesicle formation, which indicates that macropinocytosis was aborted.

The PIP3 patches that are involved in axenic macropinocytosis are a similar size to pseudopods and recruit large amounts of F-actin. Nascent macropinosomes are therefore difficult to discriminate from pseudopods, especially if they abort before vesicle formation. This is further complicated because pseudopods and macropinosomes are able to interconvert. Fig. 3 C and Video 1 show an example. The protrusion is initially free of PIP3 and resembles a normal pseudopod. At 20 s, a PIP3 patch appears at the leading edge, and shortly after, negative curvature is induced at the site of the patch. The image plainly resembles a forming macropinocytic cup. However, it does not fully close and aborts after 80 s. A kymograph of this event (Fig. 3 D) shows that the forward movement of the leading edge reduces after the appearance of PIP3.

**Suppressor of cAR mutations (SCAR) and F-actin organization in macropinosomes**

The ambiguous character of these actin structures in axenic cells complicates analysis. To more accurately distinguish the potential role of PIP3 in pseudopods and macropinosomes, we examined the behavior of the SCAR/WASP and verprolin homologue (WAVE) complex, which is the definitive marker for pseudopods. It remains associated with the leading edge throughout the progression of a pseudopod and disappears from the leading edge when the pseudopod stops advancing (Fig. 4 A; Hahne et al., 2001; Veltman et al., 2012). Interestingly, high concentrations of PIP3 do not colocalize with the SCAR complex, which is recruited to a small margin surrounding each patch (Fig. 4, B–D; and Video 2). This distribution is not only visible in the PIP3 patches that develop to form macropinosomes, but also in ones that remain planar and that cannot readily be classified as either a pseudopod or a nascent macropinosome (Video 1 and Fig. 4 B, asterisk). We were unable to find any event where a PIP3 patch was followed by recruitment of SCAR to the leading edge in the fashion that is typical for pseudopods.

A close-up view demonstrates the complexity of the macropinocytic cup. The region labeled with PIP3 remains static, with little movement perpendicular to the membrane (Fig. 4 E, green dots). SCAR remains associated with the rim of the cup throughout the event. The SCAR-containing region moves outward and drives closure of the cup (Fig. 4 E, other dots). This outward movement at the edge of the PIP3 patch can generate cell movement, though it is retarded by the slow movement at the center of the PIP3 patch. It is also clear that the edge of the patch is a major site of actin filament nucleation (Fig. 4 F). The Lifeact-mRFP signal overlies the PIP3, though it is strongest at the extremities (Fig. 4 G), and there is a clear difference between the edges and the center; the SCAR-labeled edges are rough and their thickness is variable, like pseudopods, whereas the central F-actin is thinner and smooth.

Collectively, these data indicate that PIP3 marks membrane regions that are destined for invagination during axenic growth. In axenic cells, this results in a complex pattern of inward and outward movement of the membrane, which combines with F-actin to mediate both macropinocytosis and a subset of cell movement.

**Inverse correlation between macropinocytosis and chemotaxis**

We assessed the relationship between macropinocytosis and chemotaxis by altering the macropinocytic rate. Axenically grown AX2 cells form FITC-labeled vesicles at a rate of 0.44/µm/min (Fig. 5 A). However, AX2 cells that have been cultivated on bacteria have a diminished rate of macropinocytosis. Macropinosomes in NC4 cells are even rarer, with a mean rate of 0.05/µm/min. These changes correlate with the efficiency of chemotaxis towards folate (Fig. 5 A).

Folate chemotaxis is robust under a sheet of agarose (for example Laevsky and Knecht, 2001; and Blagg et al., 2003). We examined whether the difference between under-agar and chamber assays was connected with changes in macropinocytosis. Surprisingly, macropinocytosis in vegetative axenic cells is almost completely blocked under agar, with the number of macropinosomes dropping below those in nonaxenic cells (Fig. 5 A). It is not clear why physical constraint causes such a large suppression of macropinocytosis, but it is reminiscent of the induction of autophagy by compression (King et al., 2011). However, the effect on chemotaxis is clear: axenic cells become chemotactic when macropinocytosis is blocked under agarose despite being nonchemotactic when unrestricted.

**Loss of PI 3-kinase blocks macropinocytosis and rescues migration and folate chemotaxis**

The inverse correlation between macropinocytosis and chemotaxis raises an interesting conflict. Despite the belief that PIP3 is fundamental to chemotaxis, we have found that PIP3-driven macropinocytosis is not consistent with chemotaxis to folate. We therefore examined a strain lacking all five PI 3-kinases (Hoeller and Kay, 2007). These cells have no PIP3 patches, and their axenic growth is slow due to a defect in macropinocytosis (Hoeller et al., 2013). In our hands, the rate of macropinocytosis of PI 3-kinase-null cells was only 0.05/µm/min, similar to that of nonaxenic cells. However, chemotaxis toward folate was greatly improved (Fig. 5 A). Bacterially grown AX2 migrated toward folate with a weak chemotaxis index of 0.23 and a speed of 5.6 µm/min; knocking out all the PI 3-kinases restored this to 0.44 and 19.1 µm/min, respectively (Fig. 5 B). Similarly, 10 µM LY294002, enough to inhibit PI 3-kinase without blocking the target of rapamycin (TOR) complex, significantly diminished macropinocytosis and increased cell speed (Fig. S2). Thus, it appears that PIP3 is not only inessential for folate chemotaxis in D. discoideum, but actively opposes it.

**Axenic mutations, macropinocytosis, and chemotaxis**

Our data clearly show that PIP3 patches are not used at all by cells chemotaxing to one of the two principal attractants used
by *D. discoideum* cells. Thus, at the very least, PIP₃ is not a universal player in chemotaxis. This is not a surprise, as a great deal of data has accumulated over the past six years showing that PI 3-kinases are not required for chemotaxis. What was a surprise, however, was that PIP₃ actually opposes folate chemotaxis. Folate and cAMP mostly use the same signaling by *D. discoideum* cells.
components, in particular the single G protein βγ subunit, which is thought to mediate most of the responses downstream of chemoattractant receptors. The key difference is therefore not likely to be in signaling pathways, but rather the developmental stages of the cells. While cells are growing, axenic mutations lead to massive, PIP₃-driven macropinocytosis, which is clearly inconsistent with chemotaxis. Starved axenic cells cease macropinocytosis and thus regain chemotaxis at the time they become sensitive to cAMP.

The identity of the axenic mutations is not yet known, but it will be fascinating to unravel their influence on chemotaxis at all stages. This may fundamentally alter our understanding of chemotaxis in D. discoideum and metazoan cells.

Materials and methods

Cell strains and cultivation
D. discoideum AX2 cells were used as an axenic strain and NC4 cells were used as nonaxenic, wild-type cells. The PI3 kinase quintuple knockout strain HM1200 (Hoeller and Kay, 2007) was obtained from the Dicy Stock Center. For axenic growth, cells were cultivated on Petri dishes under HL5 medium (ForMedium). Nonaxenic cultivation was done on Petri dishes under SorMC buffer supplemented with live Klebsiella aerogenes at an OD₆₀₀ of 2. SorMC buffer consists of 15 mM KH₂PO₄, 2 mM Na₂HPO₄, 50 µM MgCl₂, and 50 µM CaCl₂, pH 6.0. To reduce autofluorescence of axenically cultivated cells in preparation for fluorescence microscopy, cells were incubated overnight in LBFla medium (ForMedium). For microscopy, cells were put on a 3.5-cm acid-washed glass-bottom MatTek dish.

DNA constructs and transfection
In brief, the act6 promoter driving the resistance marker of the modular pDEM expression vector set (Veltman et al., 2009) was replaced by the coaA promoter (bp -293 to bp -1 relative to the start codon of coaA). The PIP₃-binding PH domain of cytoplasmic regulator of adenylyl cyclase (CRAC; residue 1-126 of the protein encoded by dagA) was used as a PIP₃ sensor. Full-length HSPC300 was used as a marker for the SCAR complex, and Lifeact (Riedl et al., 2008) was used as a marker for filamentos actin. Gene fragments were cloned into the BglII–SpeI site of the expression vector. For coexpression of two genes, the shuttle vector was used as described previously (Veltman et al., 2009). avGFP S65T and mCherry were used for fluorescence labeling.

Transfection of nonaxenic cells was performed as follows: 5 × 10⁶ cells were harvested from the plate, washed once in SorMC buffer, and resuspended in 400 µl SorMC buffer. Cells were mixed with 5 µl mini-prep DNA (~0.5–1 µg total) and put on ice. Cells were then electroporated at 300 V using the high-voltage setting of an ECM 399 Exponential Decay Wave Electroporation System (Harvard Apparatus) and immediately transferred to a Petri dish with SorMC buffer and live K. aerogenes at an OD₆₀₀ of 2. Selection marker was added after 5 h (10 µg/ml G418 or 100 µg/ml hygromycin).

Microscopy and image analysis
All microscopy was performed at room temperature. Confocal laser scanning microscopy was performed on a confocal microscope (A1R; Nikon) using a 1.4 NA Plan-Apochromat 60x oil immersion objective lens and the manufacturer’s NIS Elements acquisition software (Nikon). Total internal reflection fluorescence microscopy (TIRF) microscopy was performed on an inverted microscope (Eclipse TE2000U; Nikon) that was fitted with a custom TIRF condenser and a 1.45 NA 100x Plan-Apochromat TIRF objective lens. Red and green fluorescent signals were separated using a beam splitter and condenser and a 1.45 NA 100× Plan-Apochromat TIRF objective lens. Red and green fluorescent signals were separated using a beam splitter and condenser and a 1.45 NA 100× Plan-Apochromat TIRF objective lens. Red and green fluorescent signals were separated using a beam splitter and condenser and a 1.45 NA 100× Plan-Apochromat TIRF objective lens.

Fluorescence images were analyzed using the standard tools available in ImageJ. Images were not processed other than brightness/contrast adjustments and bleech correction for mRFP in Video 2. The Angle tool was used to determine the angle between a protrusion and the gradient. Graphs of the collected angle data were generated in MATLAB (MathWorks). Statistical analysis was done using the Circular Statistics Toolbox (Berens, 2009).

Chemotaxis assays
Chemotaxis assays were performed on an inverted microscope (Eclipse TE2000U) using a 10x phase contrast objective lens (NA 0.3). The microscope was controlled using Micromanager (Edelstein et al., 2010). Incubation chambers (Muinonen-Martin et al., 2010) were used to expose cells to a chemotactic gradient of 0–100 µM folate across a bridge that was 1 mm wide. Typically, a recording of 60 min was captured, starting immediately after the assembly of the chemotaxis chamber. The position of at least 20 randomly selected cells in each recording was determined every minute using the ImageJ plugin mTrackJ (Meijering et al., 2012). The chemotaxis index of the population was calculated for each time

Figure 5. PIP₃-induced macropinocytosis is incompatible with chemotaxis. (A) Negative correlation of macropinocytosis and chemotaxis. The rate of macropinosome formation under different conditions was measured using FITC-dextran and fluorescence microscopy. Each data point represents a single cell that was imaged for at least 5 min. Data were taken from at least two independent experiments. Chemotaxis is represented qualitatively to avoid quantitative comparisons made under noncomparable conditions. (B) Deletion of PI 3-kinases from axenically growing cells restores chemotaxis to folate. (B, right) Incubation chamber chemotaxis of axenic AX2 cells quintuply deleted for all p110 PI 3-kinases. (B, left) Chemotaxis of axenic wild-type cells (see data from Fig. 1 C for comparison).
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