Chapter 20

Assessment of Development and Chemotaxis in *Dictyostelium discoideum* Mutants

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Abstract

Studies using the social amoeba *Dictyostelium discoideum* have greatly contributed to the current understanding of the signaling network that underlies chemotaxis. Since directed migration is essential for normal *D. discoideum* multicellular development, mutants with chemotactic impairments are likely to have abnormal developmental morphologies. We have used multicellular development as a readout in a screen of mutants to identify new potential regulators of chemotaxis. In this chapter, we describe how mutants generated by restriction enzyme-mediated integration (REMI) are analyzed, from assessment of development to detailed characterization of 3′,5′-cyclic adenosine monophosphate (cAMP)-induced responses. Two complementary approaches, plating cells either clonally on a bacterial lawn or as a population on non-nutrient agar, are used to evaluate multicellular development. Once mutants with aberrant developmental phenotypes are identified, their chemotaxis toward cAMP is assessed by both small population and micropipette assays. Furthermore, mutants are tested for defects in both general and specific signaling pathways by examining the recruitment of actin-binding $LimE_{\Delta coil}$ or PIP3-binding PH domains to the plasma membrane in response to cAMP stimulation.

Key words: Multicellular development, Differentiation, Under buffer assay, cAR1, Small population assay, Micropipette assay, CMFDA labeling, PIP3 dynamics, Actin

1. Introduction

Chemotaxis, or the directed migration of cells up a chemical gradient, is a conserved process that occurs in many organisms, ranging from bacteria to mammals. In humans, this fundamental process is essential for embryogenesis, wound healing, and the immune response. In addition, inappropriate migration contributes to the development and progression of a number of pathological conditions, such as tumor cell metastasis and chronic

Claire M. Wells and Maddy Parsons (eds.), *Cell Migration: Developmental Methods and Protocols*,
Methods in Molecular Biology, vol. 769, DOI 10.1007/978-1-61779-207-6_20, © Springer Science+Business Media, LLC 2011

inflammation (reviewed in (1, 2)). A powerful model system for the study of chemotaxis is the social amoeba *Dictyostelium discoideum*. In this organism, chemotaxis is a vital process both at the vegetative growth stage and during a starvation-induced developmental program, during which single cells differentiate and aggregate to form a multicellular structure, the fruiting body, consisting of a stalk and a spore head.

Studies of *D. discoideum* have led to the identification of many chemotactic regulators. In differentiated cells, the predominant chemoattractant, 3′,5′-cyclic adenosine monophosphate (cAMP), binds to the G-protein-coupled cAMP receptor (cAR1) and activates several parallel pathways that trigger actin polymerization and pseudopod extension (reviewed in (3)). One key signaling molecule important for directed migration is phosphoinositide-3,4,5-trisphosphate (PIP3), produced from phosphoinositide-4,5-bisphosphate by phosphoinositide 3-kinase (PI3K) specifically at the leading edge (4, 5). PIP3 accumulation leads to the recruitment of downstream effectors, such as PKBA and cytosolic regulator of adenylyl cyclase (CRAC), that mediate actin polymerization and signal relay (6, 7). In addition, *D. discoideum* has separate PIP3-independent pathways, involving phospholipase A_2 and target of rapamycin (TOR) complex 2 (TORC2), that also contribute to actin polymerization and migration (8–10).

Despite recent insights into the network of chemotactic pathways, many signaling molecules remain unknown. One approach to identifying novel chemotactic regulators is to analyze a collection of mutant strains generated by restriction enzyme-mediated integration (REMI). In this method, a blasticidin resistance cassette is inserted at random into genomic restriction sites (e.g., DpnII) approximately once per cell, presumably disrupting the function of any gene(s) located near the insertion site (11, 12). Since directed migration is important for many stages in the *D. discoideum* developmental cycle, impaired chemotactic behavior often results in defective multicellular morphology, which provides a convenient readout that can readily be assessed in a high-throughput manner. Therefore, REMI mutants are first screened for developmental defects and are then assayed for chemotactic ability to determine whether the gene disrupted by blasticidin insertion is involved in directed migration.

In this chapter, we describe how a typical REMI mutant is analyzed, from the initial assessment of multicellular development to a detailed examination of chemotaxis. Many different approaches are available for monitoring chemotaxis, including the micropipette, small population, Zigmond chamber, under agar, and microfluidic assays. Here, we focus on the two techniques that we use most frequently in our laboratory, the small population and micropipette assays, which together assess many complementary aspects of chemotactic behavior. In addition,

mutants are tested for their ability to generate PIP3 and polymerize actin in response to cAMP stimulation by monitoring the recruitment of PIP3-binding PH domains and filamentous actin-binding proteins to the plasma membrane.

2. Materials

2.1. Assessment of Developmental Phenotypes

2.1.1. Development on a Bacterial Lawn

1. HL5 medium: 10 g/L dextrose, 10 g/L proteose peptone, 5 g/L yeast extract, 0.965 g/L $Na_2HPO_4 \cdot 7H_2O$, and 0.485 g/L KH_2PO_4 in water (see Note 1). Unless otherwise indicated, HL5 medium also contains 0.03 g/L streptomycin. Autoclave the medium and store at room temperature.
2. 10 cm Petri dish.
3. SM/5 plates: 2 g/L dextrose, 2 g/L Bacto™ peptone, 0.2 g/L yeast extract, 2.31 g/L KH_2PO_4, 1 g/L K_2HPO_4, and 20 g/L agar in water. Autoclave the mixture, allow it to cool to approximately 60°C, and pour 30 ml of the agar solution per 10-cm Petri dish. Once the agar solidifies, store the plates at 4°C for up to 1 month.
4. SM plates: 10 g/L dextrose, 10 g/L Bacto™ peptone, 1 g/L yeast extract, 2.31 g/L KH_2PO_4, 1 g/L K_2HPO_4, and 20 g/L agar in water. Autoclave, cool, and pour the agar into plates as described in Item 3 above. Store the plates at 4°C for up to 1 month.
5. *Klebsiella aerogenes* on an SM plate: Prepare a suspension of *K. aerogenes* in HL5 medium without antibiotics, spread this onto an SM plate, and grow the bacteria at room temperature overnight for use the next day. These plates can also be stored at 4°C for up to 1 month after overnight growth.

2.1.2. Development on Non-nutrient Agar

1. 10× Phosphate buffer (PB): 13.4 g/L $Na_2HPO_4 \cdot 7H_2O$ and 6.8 g/L KH_2PO_4 in water. Store the 10× stock at 4°C and dilute it to 1× with water for use.
2. Development buffer (DB): Supplement 1× PB with 2 mM $MgSO_4$ and 0.2 mM $CaCl_2$ and store at 4°C.
3. DB agar: 1.5% (w/v) agar in DB. Autoclave the mixture and store at room temperature. Prior to use, reheat it in the microwave until the agar is dissolved.
4. 6-Well tissue culture plate or 35-mm tissue-culture dish.

2.2. Preparation of *D. discoideum* cells for Chemotactic Analysis: Cell Differentiation

1. DB (see Subheading 2.1.2).
2. cAMP: Prepare a 100 mM stock solution of cAMP sodium salt monohydrate in water. Use the 100 mM stock to prepare a 1 mM working stock in water. Store aliquots at –20°C.

These stock solutions can be freeze-thawed several times, although it is not recommended to reuse less concentrated solutions. To prevent cAMP degradation by environmental phosphodiesterases, surfaces that may come into contact with the solution should not be handled directly.

3. Peristaltic pump (Gilson Minipuls 3).
4. Manifold pump tubing (Fisherbrand; Accu-Rated PVC, ID 0.76 mm) connected on both ends by the tip of a 20-G 1.5 needle to 50 cm of 0.8-mm tubing (Saint Gobain Performance Plastics; Tygon R-3603).
5. Table-top timer.

2.3. Assessment of Differentiation

1. DB (see Subheading 2.1.2).
2. 6-Well tissue-culture plate or 35-mm tissue-culture dish.

2.3.1. Under Buffer Assay

2.3.2. Cell Lysis and Immunoblotting

1. 3× Sample buffer (such as the 3× sodium dodecyl sulfate (SDS) blue loading buffer): 187.5 mM Tris–HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 125 mM DL-dithiothreitol, and 0.03% (w/v) bromophenol blue in water. Store aliquots at –20°C and dilute them to 1× in water for use (see Notes 2 and 3).
2. Criterion Tris–HCl 4–15% polyacrylamide gel.
3. Immun-Blot PVDF membrane (0.2 μm).
4. 20× TBST: 48 g/L Tris base, 160 g/L NaCl, and 2% Tween-20 in water. Adjust the pH to 7.6 and store the 20× stock at 4°C. Dilute the stock to 1× with water and store this at room temperature.
5. TBST/skim milk: 1× TBST with 5% (w/v) skim milk powder. Shake the mixture briefly until the milk powder is dissolved. Make a fresh solution prior to each use.
6. TBST/BSA: 1× TBST with 5% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) NaN_3. Shake the mixture briefly until the BSA is dissolved and store at 4°C.
7. Anti-cAR1 primary antibody from rabbit.
8. Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody from donkey.
9. ECL Western blotting detection reagent.
10. Coomassie brilliant blue (CBB) staining solution: 40% (v/v) methanol, 10% (v/v) glacial acetic acid, and 0.1% (w/v) CBB in water. Store this at room temperature.
11. Destaining solution: 40% (v/v) methanol and 10% (v/v) glacial acetic acid in water. Store this at room temperature.

2.4. Analysis of Chemotaxis By Scoring or Video Acquisition of a Small Population Assay

2.4.1. Scoring Method for the Small Population Assay

1. 1× PB (see Subheading 2.1.2).
2. 10-cm Petri dish filled with noble agar: 1% (w/v) solution of Difco™ noble agar (BD Biosciences) in 1× PB. Boil the mixture until the agar is dissolved, allow it to cool slightly, and pour 10 ml of the agar solution per 10-cm Petri dish. Cover the plate and allow the agar to solidify for 30–60 min prior to use (see Note 4).
3. Drawn out Pasteur pipettes: Draw out 9″ glass Pasteur pipettes by holding them at each end so that the thin end of the pipette is over a flame. When the glass becomes pliable, pull the two ends apart to extend the thin portion of the pipette into a capillary. Cut off the very tip of the capillary using a razor blade to make a small opening.
4. 1×10^{-8} M, 1×10^{-7} M, and 1×10^{-6} M cAMP: Dilute the 1 mM cAMP stock solution (see Subheading 2.2) in 1× PB. Make fresh dilutions prior to each use.
5. Developed cells (see Subheading 3.2).

2.4.2. Video Acquisition of the Small Population Assay

1. 1× PB (see Subheading 2.1.2).
2. 1-well chambered coverglass filled with noble agar: 1% (w/v) solution of Difco™ noble agar (BD Biosciences) in 1× PB. Boil the mixture until the agar is dissolved, allow it to cool slightly, and pour 2 ml of the agar solution per 1-well Lab-Tek™ chambered coverglass (Thermo Scientific, Nunc). Allow the agar to solidify for 15–30 min prior to use (see Note 4).
3. 1×10^{-6} M cAMP: Dilute the 1 mM cAMP stock solution (see Subheading 2.2) in 1× PB.
4. Developed cells (see Subheading 3.2).

2.5. Analysis of Chemotaxis by a Micropipette Assay

2.5.1. Analysis of Unlabeled Cells

1. DB (see Subheading 2.1.2).
2. Femtotips.
3. Microloader tips.
4. FemtoJet microinjector (Eppendorf).
5. 1×10^{-5} M cAMP: Dilute the 1 mM cAMP stock solution (see Subheading 2.2) in DB that has been passed through a 0.22-μm filter.
6. 1-well Lab-Tek™ chambered cover glass (Thermo Scientific, Nunc).
7. Developed cells (see Subheading 3.2).

2.5.2. Simultaneous Analysis of Two Cell Lines Using CMFDA Labeling

1. 1× PB (see Subheading 2.1.2).
2. DMSO.

3. CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes): Prepare a 10 mM stock solution of CMFDA in DMSO. Store aliquots at −80°C.
4. Items 1–7 from Subheading 2.5.1.

2.6. Examination of PIP3 and Actin Dynamics in Chemotaxing Cells

1. DB (see Subheading 2.1.2).
2. 1×10^{-5} M cAMP: Dilute the 1 mM cAMP stock solution (see Subheading 2.2) in DB.
3. 8-well Lab-Tek™ chambered cover glass (Thermo Scientific, Nunc).
4. Developed cells (see Subheading 3.2) expressing GFP-tagged versions of either the PH-domain from CRAC (PH_{CRAC}) or a fragment of the filamentous actin-binding protein LimE ($LimE_{\Delta coil}$) for analysis of PIP3 or actin dynamics, respectively (5, 13).

2.6.1. Analysis of PIP3 and Actin Dynamics in Globally Stimulated Cells: Jumping Assay

2.6.2. Analysis of PIP3 and Actin Dynamics in a Gradient: Micropipette Assay

1. Developed cells (see Subheading 3.2) expressing GFP-tagged versions of either PH_{CRAC} or $LimE_{\Delta coil}$ (see Subheading 2.6.1).
2. Items 1–6 from Subheading 2.5.1.

3. Methods

3.1. Assessment of Developmental Phenotypes

In order to isolate strains with potential chemotactic defects from a population of REMI insertional mutants, cells are assessed for their ability to enter into the developmental program and form multicellular structures. One way to examine development is to clonally plate a population of REMI mutants on a bacterial lawn. Under these conditions, single vegetative cells proliferate as they feed on the bacteria, resulting in circular plaques that contain cells at every stage of the *D. discoideum* life cycle (reviewed in ref. 14). In the center of the plaque, where the bacterial food source has been completely depleted, the cells starve and initiate a developmental program in which they differentiate, aggregate, and ultimately form fruiting bodies (Fig. 1a). Further from the center of the plaque, cells undergo starvation later and, therefore, represent progressively earlier stages of the life cycle, including the mounding and streaming stages. At the very edge of the plaque, cells continue to feed on bacteria and remain in the vegetative state. Aberrant development leads to the enrichment of cells in particular stages of the life cycle and abnormal multicellular morphology, ranging from a complete inability to aggregate to an arrest at the streaming or mounding stages or to the formation of abnormal fruiting bodies. In addition, plaque morphology can be distinguished based on size, the shape of the border, and the extent to which the bacterial lawn is cleared by the cells before

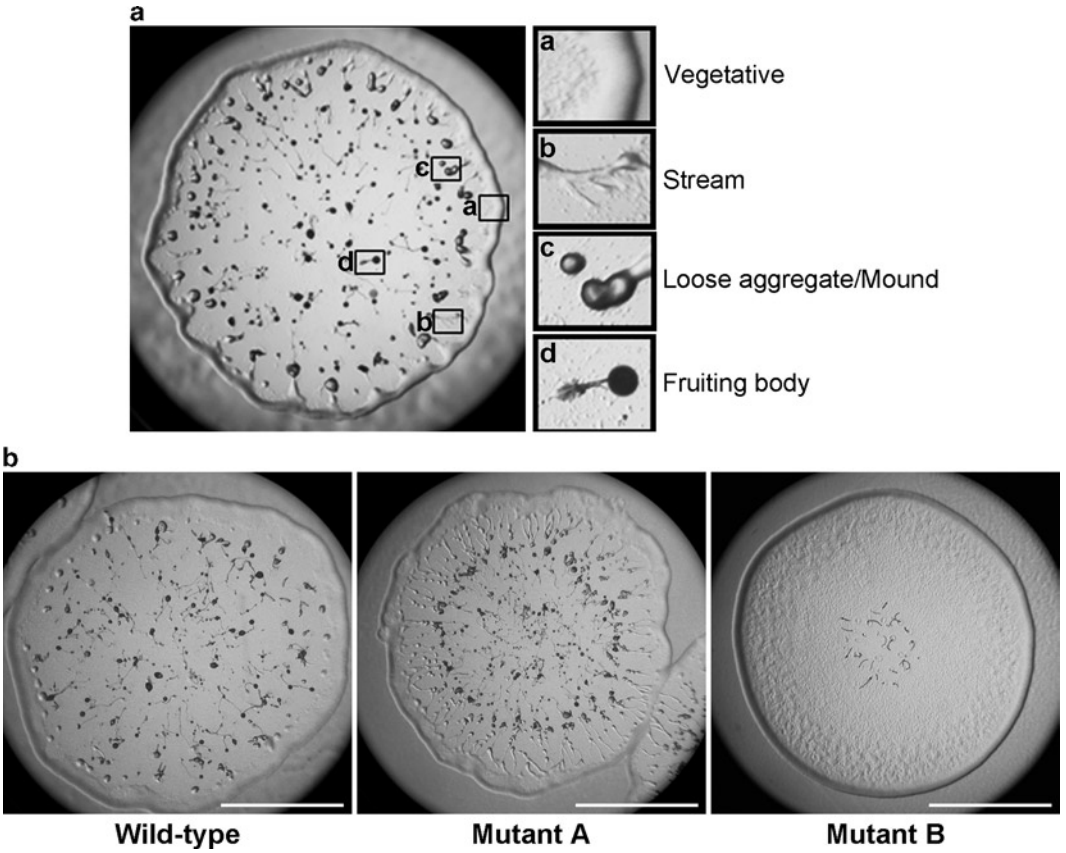

Fig. 1. *Dictyostelium discoideum* cells develop on a bacterial lawn. Single cells were plated clonally on a lawn of *K. aerogenes* and grown at room temperature for 4 days before the resulting plaques were photographed under a dissection microscope. (**a**) A representative wild-type plaque showing the various developmental stages of the *D. discoideum* life cycle. (**b**) Comparison of plaque morphology of wild-type and two mutant cell lines. Mutant A cells arrest at the streaming stage of development, whereas mutant B cells fail to aggregate completely. Scale bar, 5 mm.

they expand outward or undergo starvation. Figure 1b shows representative wild-type, stream-arrest, and aggregation-less plaques.

D. discoideum development can also be assessed by plating a population of cells on non-nutrient agar. In this assay, cells synchronously enter into the developmental program without proliferation, such that the entire population of cells represents the same stage of the life cycle at any given moment. This provides a useful tool by which mutant strains can be monitored over time as they progress through the developmental program.

3.1.1. Development on a Bacterial Lawn

1. Collect *D. discoideum* cells in the exponential growth phase (see Note 5) and count the cells using a hemocytometer. Dilute cell suspension to a final concentration of approximately 2 cells/μl in HL5 medium without antibiotics.

2. Prepare a suspension of *K. aerogenes* by collecting the bacteria from an SM plate using a sterile loop and resuspending the cells in HL5 medium without antibiotics. The bacterial suspension should be opaque (see Note 6).
3. Pipet 260 µl of the *K. aerogenes* suspension into the center of a room temperature SM/5 plate (see Note 7). Add 15–30 µl of the diluted *D. discoideum* cells to the bacteria and use a sterile spreader to distribute the cells uniformly across the plate. It is recommended to make multiple plates, each with a different volume of the diluted *D. discoideum* cells, to ensure that roughly 30–60 individual plaques will grow without touching each other on at least one plate. Avoid moving the plates until the liquid is absorbed by the agar. Keep the plates at room temperature overnight and invert them the next day. Continue to keep the plates at room temperature.
4. Four days after plating the cells, the resulting plaques can be analyzed for developmental phenotypes. Acquire images of representative plaques using a dissection microscope equipped with a camera. By the fourth day, wild-type cells form round plaques with large fruiting bodies in the center and vegetative cells around the periphery.

3.1.2. Development on Non-nutrient Agar

1. Collect *D. discoideum* cells in the exponential growth phase (see Note 5) and count the cells using a hemocytometer.
2. Melt the DB agar in the microwave, allow it to cool slightly, and pour 3 ml of the agar solution per well of a 6-well tissue-culture plate (or per 35-mm tissue-culture dish). Allow the agar to solidify for 1 h with the lid slightly off-set from the plate.
3. Centrifuge 2×10^7 cells in a 15-ml polypropylene tube at 360 rcf for 3–4 min. Discard the supernatant. To wash, resuspend the cell pellet in 15 ml of DB by gently vortexing and/or repeatedly pipetting the cells; repeat the centrifugation and discard the supernatant. Resuspend the resulting pellet in 1 ml of DB for a final density of 2×10^7 cells/ml.
4. Dilute the cells in 1 ml of DB, using at least two different densities for each cell line. Typically, densities of 1×10^6 cells/ml and 5×10^6 cells/ml are used; however, 1×10^7 cells/ml can also be used.
5. Plate 1 ml each of the diluted cells onto the solidified DB agar. Allow the cells to attach for approximately 10 min. Tilt the plate and aspirate the liquid. Keep the plate tilted and aspirate any remaining liquid after approximately 1 min. Leave the plate with an off-set lid until the cells acquire a "matte" finish, for approximately 20 min. Close the lid and leave the plate at room temperature until the cells reach the desired developmental stage.

6. Monitor the progression of the cells through the developmental program using a dissection microscope equipped with a camera to acquire images of the desired stage(s) of the life cycle. Wild-type cells typically begin streaming approximately 5 h after plating and form fruiting bodies within 24 h.

3.2. Preparation of D. discoideum Cells for Chemotactic Analysis: Cell Differentiation

Once mutants with aberrant developmental phenotypes have been identified, they are then assessed for defects in chemotaxis. Since vegetative cells are not responsive to cAMP, the cells must be differentiated prior to chemotactic analysis. This can be achieved by inducing the upregulation of a variety of developmental genes, including the cAMP receptor cAR1, through a combination of starvation and the addition of cAMP at regular intervals (15).

1. Collect *D. discoideum* cells in the exponential growth phase (see Note 5) and count the cells using a hemocytometer.
2. Centrifuge 8×10^7 cells in a 50-ml polypropylene tube at 360 rcf for 3–4 min. Discard the supernatant. To wash, resuspend the pellet in 50 ml of DB by gently vortexing and/or repeatedly pipetting the cells; repeat the centrifugation and discard the supernatant. Wash the pellet once more with 50 ml of DB. Resuspend the resulting pellet in 4 ml of DB for a final density of 2×10^7 cells/ml (see Note 8).
3. Transfer the cell suspension to a 50-ml Erlenmeyer flask (see Note 8). Initiate starvation by shaking the flask at 115 rpm on a rotary shaker for 1 h.
4. Connect one piece of tubing for each cell line to the peristaltic pump and wash the tubing with water or DB. Fill the tubing with a solution of 2 μM cAMP in DB, such that the final cAMP concentration is 50 nM when 100 μl of the cAMP solution is added to the cell suspension (see Note 9). Without turning on the pump, suspend one end of the tubing above each flask of cells and secure it with tape. Keep the other end of the tubing submersed in the cAMP solution.
5. Connect the peristaltic pump to a timer so that the pump will turn on for 5 s at 6-min intervals. The pump should be calibrated to release 100 μl of the cAMP solution with each pulse (every 6 min).
6. After 1 h of starvation, turn on the peristaltic pump to begin pulsing the cells with cAMP. Continue to shake the cells at 115 rpm on a rotary shaker. Wild-type cells are typically differentiated and ready for analysis after 4 h of cAMP pulsing (or 5 h of starvation).

3.3. Assessment of Differentiation

This section describes two assays for determining the extent of cell differentiation (see Subheading 3.2) prior to conducting assays of chemotaxis. Because cells are only competent to respond to cAMP

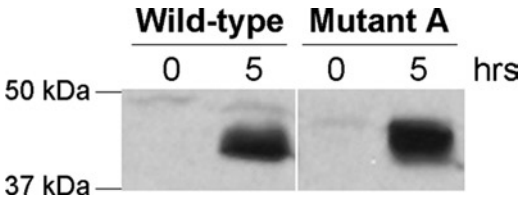

Fig. 2. cAMP receptor (cAR1) protein levels increase during the course of cell differentiation. An equal number of wild-type or mutant A cells were collected and lysed at 0 or 5 h after development was initiated. Proteins from approximately 1.5×10^6 cells were separated by SDS-PAGE and immunoblotted using an anti-cAR1 antibody. No cAR1 protein is observed for either cell line before development (0 h). However, a band corresponding to the endogenous cAR1 protein is observed at comparable levels for both wild-type and mutant A cells 5 h after development.

when they are differentiated, variability in the state of differentiation across cell lines may affect the results of chemotactic analyses. The "under buffer" assay is a method that allows for the rapid assessment of cell differentiation. Fully differentiated cells acquire an elongated and polarized morphology, form streams, and eventually aggregate when plated on a tissue-culture dish filled with buffer. Observing this behavior serves as a good indication that cells are differentiated and ready for chemotactic analysis.

Another method of assessing differentiation is to examine the expression of genes, such as cAR1, that are upregulated during development. To do this, cells are lysed and assessed for cAR1 expression, using protein levels as a readout, both before and after differentiation. Figure 2 shows a representative immunoblot of endogenous cAR1 protein levels for the wild-type and mutant A cell lines depicted in Fig. 1b. Wild-type cells show a dramatic increase in cAR1 protein levels after 5 h of starvation. Although mutant A has aberrant development on a bacterial lawn, these cells are capable of differentiating, as indicated by wild-type-like cAR1 levels, in response to pulses of exogenous cAMP. This suggests that any impairments observed for this mutant strain during chemotactic analysis will not be caused by defects in cell differentiation.

3.3.1. Under Buffer Assay

1. 5 h after initiating development (see Subheading 3.2) or prior to assaying chemotaxis, add 3.5×10^6 cells to 3 ml of DB in a well of a 6-well tissue culture plate. Pipet the cells up and down several times to dissociate any clumps.
2. Observe the behavior of the cells using an inverted microscope. Typically, differentiated wild-type cells will form streams within 15–30 min of plating and form tight aggregates after 2–3 h.

3.3.2. Cell Lysis and Immunoblotting

1. At the desired times after initiating development (see Subheading 3.2), collect 4×10^6 cells in a 1.5-ml Eppendorf tube (see Note 10). Centrifuge the cells at 300 rcf for 2–3 min.

2. Aspirate the supernatant and add 40 µl of 1× sample buffer to lyse the cells. Resuspend the pellet by vortexing. At this point, the lysates can either be analyzed directly by immunoblotting or stored at −20°C for analysis at a later date (see Note 11).
3. Load 10–15 µl of the cell lysates onto a 4–15% gradient polyacrylamide gel. Run the gel at 200 V for 60 min or at 150 V for 90 min.
4. Transfer the proteins onto a PVDF membrane at 75 V for 1 h 15 min.
5. Rinse the membrane with 1× TBST and incubate with a blocking solution of TBST/skim milk. Shake the membrane on a rotary shaker for at least 30 min at room temperature or overnight at 4°C. Rinse with 1× TBST after blocking.
6. Incubate the membrane with a 1:1,000 dilution of the primary antibody (anti-cAR1 from rabbit) in TBST/BSA. Shake the membrane on a rotary shaker for 1 h at room temperature (see Notes 12 and 13).
7. Wash the membrane with 1× TBST twice for 10 min each and incubate it with a 1:5,000 dilution of the secondary antibody (HRP-conjugated anti-rabbit) in TBST/skim milk. Shake the membrane on a rotary shaker for 45–60 min at room temperature.
8. Wash the membrane with 1× TBST once for 10 min and three times for 5 min each and detect by chemiluminescence using ECL Western blotting detection reagent. In wild-type cells, the cAR1 antibody specifically recognizes a band at approximately 40 kDa in developed (5 h) but not vegetative (0 h) cells. Occasionally, a size shift will be observed for this band, depending on the phosphorylation state of the cAR1 receptor at the particular moment that the cell samples were prepared.
9. To confirm equal sample loading, stain the membrane with Coomassie brilliant blue staining solution for approximately 10 min and then rinse it with water a few times. Wash the membrane with destaining solution until the desired contrast is achieved (see Note 14). Air-dry the membrane.

3.4. Analysis of Chemotaxis By Scoring or Video Acquisition of a Small Population Assay

One technique used to assess chemotaxis is the small population assay. In this assay, a drop of differentiated cells is placed next to a drop of cAMP on a hydrophobic agar surface. As the cAMP diffuses through the agar, it forms a gradient that causes the cells to migrate toward it. Since the cells cannot cross the boundary of their drop, they accumulate at the edge facing the cAMP. The extent of cell accumulation can be scored over time and compared between cell lines. Because of its simplicity, the small

population assay is a convenient way to assess the chemotactic ability of multiple cell lines simultaneously at several different cAMP concentrations. Furthermore, the assay can be easily adapted for time-lapse microscopy, allowing the chemotaxis of individual cells to be tracked and quantified for several cell lines at once. For example, video acquisition of the small population assay was used to compare chemotaxis for wild-type and mutant A cells (Fig. 3). The cell behaviors that occurred during image

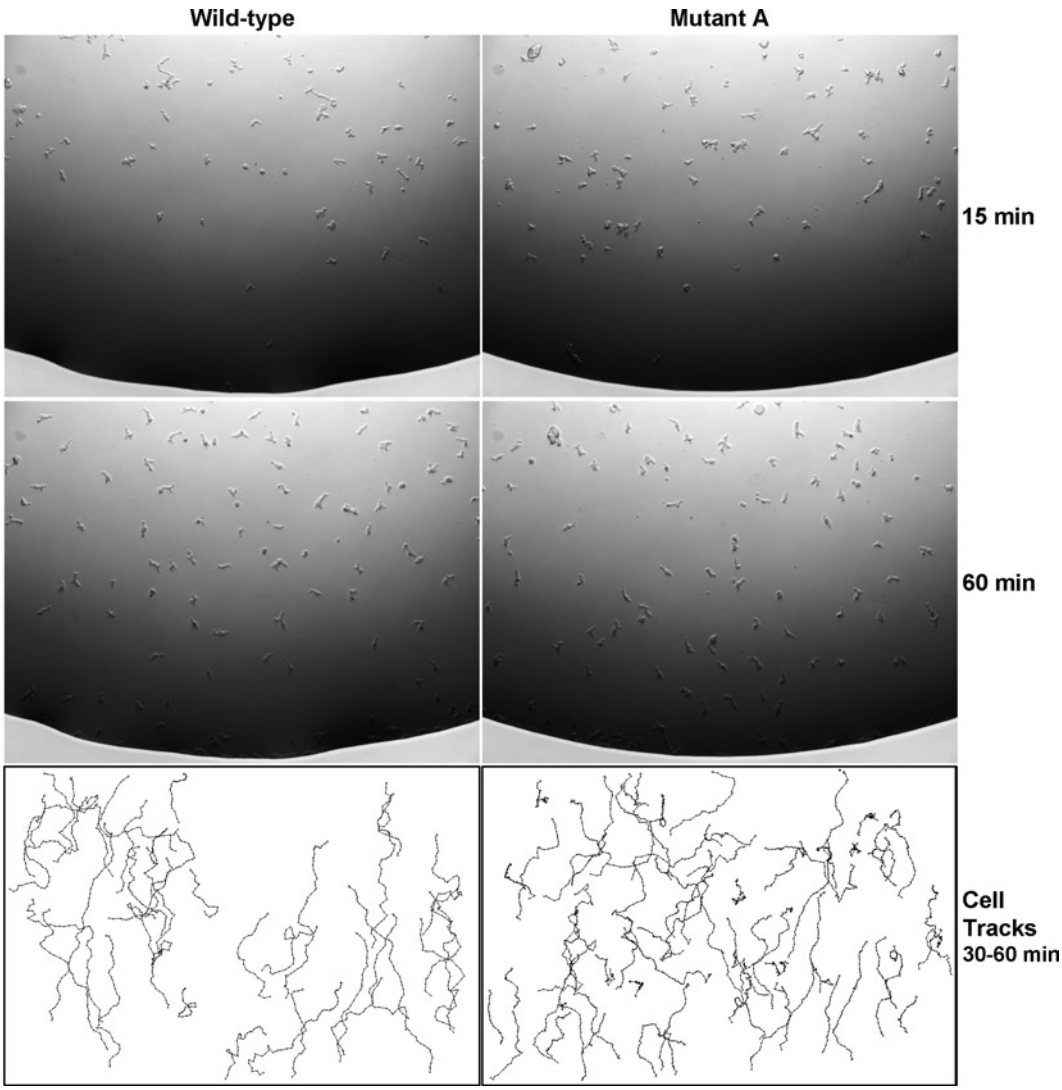

Fig. 3. The small population assay shows that mutant A cells have reduced speed during chemotaxis compared to wild-type cells. Drops of cells developed for 5 h were placed next to drops of 1 μM cAMP on a hydrophobic agar surface. Images of the cell drop borders facing the cAMP drops were acquired with a 10× objective and DIC illumination at 30-s intervals until 1 h after the drops were prepared. Frames taken 15 and 60 min after the drops were prepared are shown for both wild-type and mutant A cells. Tracks of the centroid of individual cells that migrated for at least 25 consecutive frames were obtained using Chemotaxis Quantification software (Version 1.2). Only frames acquired during the last 30 min of imaging were analyzed.

acquisition were quantified, demonstrating that mutant A has reduced chemotactic speed compared to wild-type cells (4.8 vs. 8.8 μm/min), whereas its chemotactic index and persistence are not significantly affected. One of the disadvantages of this assay is that cells are imaged at a low magnification, such that differences in cell morphology cannot easily be observed.

3.4.1. Scoring Method for the Small Population Assay

1. 5 h after initiating development (see Subheading 3.2), or when cells prepared for the under buffer assay appear differentiated (see Subheading 3.3.1), dilute the cells to a density of 1×10^6 cells/ml in 1× PB. Gently vortex the cells and pipet them up and down to dissociate any clumps.
2. Load the diluted cells into a drawn-out Pasteur pipette. Touch the pipette tip to the surface of a Petri dish filled with solidified noble agar to spot 10–15 small drops (approximately 1.5 μl each) of cells in a row, 3 mm apart from each other (see Note 15).
3. Use another drawn-out Pasteur pipette to spot a row of cAMP drops 3 mm below the row of cell drops. Typically, several different cAMP concentrations, including 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} M, are used for each cell line. These can be tested on the same plate by spotting paired drops of cells and cAMP in separate rows for each cAMP concentration, such that the sets of pairs are spaced approximately 1 cm apart from each other. Alternatively, different cell lines at the same cAMP concentration can be tested on the same plate.
4. Use an inverted microscope with a 10× objective to examine the bottom border of each cell drop (facing the corresponding cAMP drop). Score the response of each cell drop as follows:

   No response (−): no cells have accumulated at the border of the drop

   Intermediate response (+/−): a small fraction of the cells has accumulated at the border of the drop

   Positive response (+): a large fraction of the cells has accumulated at the border of the drop, forming a continuous line of cells piled on top of each other

   Figure 4 shows a representative image for each of the three responses. For mutant analysis, scoring should be done at 15-min intervals for 1 h.
5. Calculate the relative chemotaxis score for each cell line as follows: [number of (+/−) drops × 0.5 + number of (+) drops]/total number of drops analyzed × 100%. The score for wild-type cells should be nearly 100% by the 1-h timepoint, since all or most of the drops should show a positive response marked by a large accumulation of cells at the border of the drop facing the cAMP gradient.

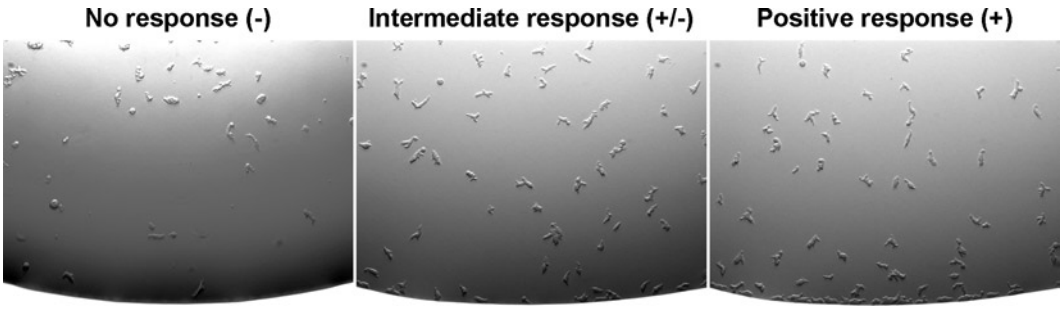

Fig. 4. Scoring for the small population assay can be used to assess chemotaxis. Drops of wild-type cells developed for 5 h were placed next to drops of 1 μM cAMP on a hydrophobic agar surface. Images of the cell drop borders facing the cAMP drops were acquired with a 10× objective and a 1.6× optovar using DIC illumination. Representative images are shown for drops with no response (–), an intermediate response (+/–), or a positive response (+).

3.4.2. Video Acquisition of the Small Population Assay

1. 5 h after initiating development (see Subheading 3.2), or when cells prepared for the under buffer assay appear differentiated (see Subheading 3.3.1), dilute the cells to a density of 6.7×10^5 cells/ml in 1× PB. Gently vortex the cells and pipet them up and down to dissociate any clumps.
2. Using a Pipetman for consistency, spot 1.5 μl drops of cells approximately 3 mm apart from each other on a 1-well chambered cover glass filled with solidified noble agar. Up to seven drops can fit onto a single 1-well chambered cover glass, allowing for the analysis of six different mutant strains and a wild-type control at the same time.
3. Spot 1.5 μl drops of cAMP in a row 3 mm below the cell drops. Typically, cAMP is used at a concentration of 1×10^{-6} M, although other concentrations can be tested as well.
4. Film the bottom edge of each cell drop (facing the corresponding cAMP drop) using an inverted microscope equipped with a moving stage and a camera (see Note 16). Images should be acquired using a 10× objective and DIC illumination at 30-s intervals for 1 h. During the course of the assay, wild-type cells migrate with directional persistence toward the border of the drop facing the cAMP gradient with a speed of approximately 10 μm/min.
5. The acquired images can be analyzed using Chemotaxis Quantification software (Version 1.2), which measures various parameters, including chemotactic speed, chemotactic index, and directional persistence (16). Typically, the last 30 min of the time-lapse acquisition (images acquired between 30 and 60 min after spotting the cells onto the agar) is analyzed.

3.5. Analysis of Chemotaxis By a Micropipette Assay

The micropipette assay is another way to analyze chemotaxis. In this assay, cells are imaged over time as they migrate toward a point source of cAMP, allowing for a detailed examination of the

morphology and behavior of individual cells. A major disadvantage of this assay is that only one field of cells can be imaged at a time. To bypass this limitation, two cell populations can be differentially labeled, mixed together, and analyzed simultaneously. For example, one strain of cells can be labeled using the membrane-permeable fluorescent dye CMFDA, while a second strain can be treated with DMSO as a control (17). Figure 5 shows that

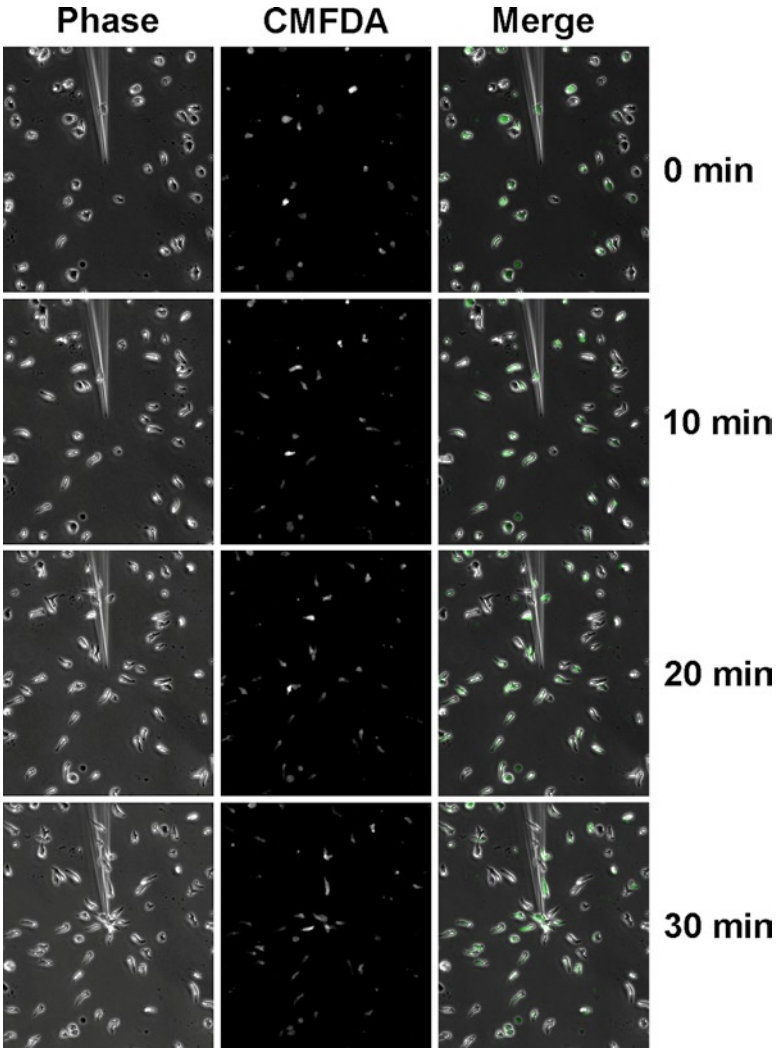

Fig. 5. Labeling with CMFDA does not affect the chemotaxis of wild-type cells. Cells developed for 5 h were labeled with CMFDA or treated with DMSO. An equal number of CMFDA-labeled and DMSO-treated cells were mixed, plated on a chambered cover glass, and exposed to a micropipette filled with 10 μM cAMP. Cells surrounding the micropipette were imaged at 30-s intervals with a 10× objective using both a fluorescein filter set and phase illumination. Sequential phase and epifluorescent images were overlaid at the indicated times, with labeled cells pseudo-colored green. After 30 min of exposure to the cAMP gradient, both labeled and unlabeled cells are able to reach the micropipette, suggesting that treatment with CMFDA does not affect chemotaxis.

wild-type cells labeled with CMFDA or treated with DMSO migrate equally well toward cAMP.

3.5.1. Analysis of Unlabeled Cells

1. 5 h after initiating development (see Subheading 3.2), or when cells prepared for the under buffer assay appear differentiated (see Subheading 3.3.1), dilute the cells to a few different densities (ranging from 1×10^5 to 4×10^5 cells/ml) in DB. Gently vortex the cells and/or pipet them up and down to dissociate any clumps (see Note 17).
2. Pipet 20–25 μl of the diluted cell suspensions onto the surface of a 1-well chambered cover glass. Several drops, of different cell densities and/or different cell lines, can be placed in the same chamber, spaced a few centimeter apart from each other. Allow the cells to adhere for 5–10 min before filling the chamber with 3–4 ml of DB.
3. Fill a femtotip (micropipette) with 1×10^{-5} M cAMP using a microloader tip and attach it to the microinjector system (see Note 18).
4. View the cells in the chambered cover glass with an inverted microscope to find a field appropriate for imaging. The field used for imaging should include enough cells for analysis, but not so many cells that they will come into contact with each other during the course of the assay. In addition, this field should include a central space for the micropipette that is mostly devoid of cells. Place the micropipette in the center of the field. Using the microinjector, allow the cAMP to flow from the micropipette tip with a compensation pressure of 30 hPa (see Note 19).
5. Image the cells using a 10× or 20× objective and either DIC or phase illumination. Acquire images every 20–30 s for a total of 30 min. During the course of the assay, wild-type cells migrate with directional persistence up the cAMP gradient, eventually reaching the tip of the micropipette. The resulting images can be analyzed using Chemotaxis Quantification software (Version 1.2), which measures various parameters, including chemotactic speed, chemotactic index, and directional persistence (16). Alternatively, cells can be imaged at a higher magnification to examine cell morphology in detail.

3.5.2. Simultaneous Analysis of Two Cell Lines Using CMFDA Labeling

1. 5 h after initiating development (see Subheading 3.2), or when cells prepared for the under buffer assay appear differentiated (see Subheading 3.3.1), centrifuge 2×10^6 cells at 300 rcf for 2 min and aspirate the supernatant. To wash, resuspend the cell pellet in 1× PB by gently vortexing and/or repeatedly pipetting the cells; repeat the centrifugation and discard the supernatant. Resuspend the pellet in 100 μl of 1× PB that contains either 100 μM CMFDA or DMSO. Incubate the cell

suspension at room temperature for 20–30 min, intermittently flicking the tube to mix the contents. Note that the cell suspension treated with CMFDA will gradually turn green.

2. Centrifuge the cell suspension and aspirate the supernatant. To wash, resuspend the pellet in 1× PB by gently vortexing and/or repeatedly pipetting the cells; repeat the centrifugation and discard the supernatant. Repeat the wash step once more. Resuspend the resulting pellet in 1× PB to obtain a final cell density of 1×10^6 cells/ml.
3. Dilute the cells to a few different final densities (ranging from 1×10^5 to 4×10^5 cells/ml) in DB, mixing equal volumes of CMFDA- and DMSO-treated cells. Proceed with steps 2–5 of the micropipette assay as described above (see Subheading 3.5.1); however, to visualize both cell populations, images should be acquired at each timepoint using both DIC or phase illumination and epifluorescence with a fluorescein filter set. Wild-type cells migrate toward the micropipette tip with similar directional persistence and speed when treated with either CMFDA or DMSO (see Fig. 5).

3.6. Examination of PIP3 and Actin Dynamics in Chemotaxing Cells

Chemoattractant stimulation leads to the rapid production of PIP3 at the membrane (5). In globally stimulated cells, this accumulation occurs transiently and uniformly around the cell membrane and is followed by a second, more prolonged peak in PIP3 production that corresponds to the generation of pseudopodia randomly around the cell periphery. In cells experiencing a chemotactic gradient, PIP3 production occurs specifically at the leading edge and is persistently maintained (4, 6, 18). In both cases, the accumulation of PIP3 results in the recruitment of PH domain-containing effector proteins, such as PKBA and CRAC, to the membrane (6, 7). These effectors, together with independent signaling pathways, lead to the polymerization of actin with temporal and spatial dynamics similar to those of the PIP3 response (18). Therefore, mutants can be assessed for defects in known chemotactic signaling pathways by monitoring the spatial and temporal changes in PIP3 production and actin polymerization that occur in response to cAMP stimulation. To do this, fluorescence time-lapse microscopy can be used to monitor cells that express GFP-tagged versions of either the PH domain from CRAC (PH_{CRAC}) or a fragment of the filamentous actin-binding protein LimE ($LimE_{\Delta coil}$), which serve as readouts for PIP3 production and actin polymerization, respectively (5, 13). Other proteins that bind PIP3 or actin with high affinity can also be fluorescently tagged and used for this assay. Figure 6 shows the PIP3 response of wild-type cells expressing PH_{CRAC}-GFP after global stimulation with cAMP.

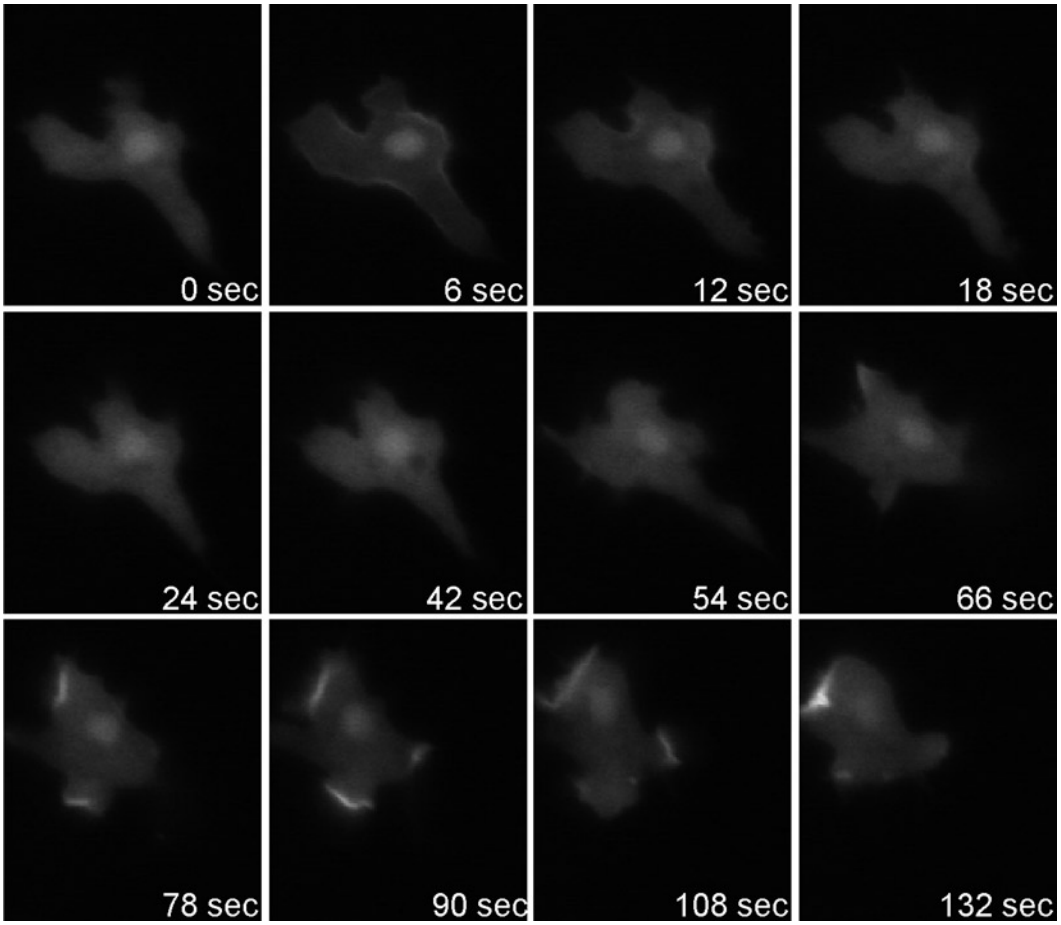

Fig. 6. PIP3 is produced in two phases upon cAMP stimulation. Wild-type cells expressing PH_{CRAC}-GFP as a readout for PIP3 levels were developed for 5 h, plated on a chambered cover glass, and imaged using an inverted microscope with a 40× oil objective. Epiflourescent images were acquired at 6-s intervals using a GFP filter set. Frames are shown for representative timepoints as indicated. After the second frame ($t = 0$ s) was acquired, the cells were stimulated globally with 1 μM cAMP, resulting in an initial burst of PIP3 production and the rapid, transient recruitment of PH_{CRAC}-GFP to the cell membrane. This initial response is followed by a second, prolonged peak in PIP3 production with a corresponding enrichment of PH_{CRAC}-GFP on membrane protrusions.

3.6.1. Analysis of PIP3 and Actin Dynamics in Globally Stimulated Cells: Jumping Assay

1. Develop cells (see Subheading 3.2) that express GFP-tagged versions of either PH_{CRAC} (PIP3 dynamics) or $LimE_{\Delta coil}$ (actin dynamics). 5 h after initiating development, or when cells prepared for the under buffer assay appear differentiated (see Subheading 3.3.1), dilute cells to a final density of 1.5×10^5 cells/ml in 10 ml of DB. Gently vortex the cells and/or pipet them up and down to dissociate any clumps.
2. Seed 450 μl of the diluted cells into each well of an 8-well chambered cover glass (for several replicates). Let the cells adhere to the chamber for approximately 5–10 min.
3. Image the cells with an inverted microscope using either a 40× or a 60× oil objective. Acquire images at 5-s intervals for

30 frames (for a total of 2.5 min) using both DIC or phase illumination and epifluorescence with a GFP filter set at each timepoint. After imaging the basal state for two frames, stimulate the cells by adding 50 µl of 10 µM cAMP to the chamber for a final cAMP concentration of 1 µM (see Note 20). Upon stimulation, wild-type cells expressing fluorescently tagged PH_{CRAC} or $LimE_{\Delta coil}$ show a robust, transient recruitment of the GFP signal from the cytosol to the membrane, followed by a prolonged period during which the GFP signal is present at the tips of pseudopodia and membrane ruffles (see Fig. 6).

3.6.2. Analysis of PIP3 and Actin Dynamics in a Gradient: Micropipette Assay

1. Develop cells (see Subheading 3.2) that express GFP-tagged versions of either PH_{CRAC} (PIP3 dynamics) or $LimE_{\Delta coil}$ (actin dynamics). Proceed with steps 1–4 of the micropipette assay as described above (see Subheading 3.5.1).
2. Image the cells with an inverted microscope using a 40× oil objective. Acquire images every 20–30 s for a total of 20–30 min, using both DIC or phase illumination and epifluorescence with a GFP filter set at each timepoint. Wild-type cells expressing fluorescently tagged PH_{CRAC} or $LimE_{\Delta coil}$ show a pronounced and persistent enrichment of the GFP signal at the leading edge as they migrate toward the micropipette tip.

4. Notes

1. The water used in all cases is deionized and double distilled.
2. To ensure a uniform solution, place the sample buffer (3× or 1×) at 37°C for a few minutes and vortex it prior to use.
3. Other denaturing sample buffer recipes can also be used.
4. The agar plates should be made immediately prior to use since they can become dehydrated, causing the drops of cells and cAMP to dry out.
5. Grow *D. discoideum* axenically, without bacteria, at 22°C in HL5 medium. When preparing for experiments, grow cells in suspension culture at 200 rpm on a rotary shaker. Start a suspension culture so that the initial density is at least ~10^5 cells/ml. Experiments should be conducted using cells in the exponential growth phase, between 2×10^6 and 6×10^6 cells/ml. The length of time required to culture the cells to the necessary density can be estimated based on a doubling time of 8–12 h as observed for wild-type strains. For long-term maintenance, grow the cells on tissue-culture plates. Although this method does not provide as many cells for use in experiments, it provides better growth conditions compared to suspension

culture. For strains that cannot grow in suspension, for example, many mutants with cytokinesis defects, adherent cells from tissue-culture plates can also be used for experiments. In this case, aspirate the HL5 medium, add approximately 3 ml of fresh medium, and detach the cells from the surface of the plate by repeat pipetting. Collect the cell suspension from the plate and proceed as described for cells in a suspension culture. Unless otherwise indicated, all experiments are carried out at room temperature.

6. To prepare a source of bacteria, *K. aerogenes* cells can also be grown in suspension culture in HL5 medium without antibiotics overnight at room temperature for use the next day.
7. SM plates can be used instead of SM/5. In this case, plaques are analyzed 5 days after plating the cells. Additionally, the rate of plaque growth and the rate at which cells enter into the developmental program can be altered by plating more or less of the *K. aerogenes* suspension for the bacterial lawn.
8. The total number of cells used for differentiation can be altered as long as the volume used for resuspension is adjusted accordingly to maintain a final cell density of 2×10^7 cells/ml. For example, if 4×10^7 cells are used, they should be resuspended in 2 ml of DB. Cell volumes of less than 1 ml or greater than 10 ml should be avoided if possible. For volumes of 3 ml or less, 25-ml Erlenmeyer flasks should be used.
9. Final cAMP concentrations of 75–100 nM can also be used. The concentration of the cAMP solution must be adjusted if the volume of the cell suspension is altered (see Note 8). The stated concentration of 2 μM cAMP is based on a cell suspension volume of 4 ml.
10. For a full developmental timecourse, samples are typically prepared 0, 3, 4, 5, 6, and 7 h after starvation is induced. Alternatively, samples can be taken both at 0 h and immediately before conducting an experiment, usually at 5 h, to confirm that the cells being assessed have differentiated. When calculating the volume of the cell suspension to be removed at each timepoint, changes in cell density over time due to the addition of cAMP (1 ml every hour) should be taken into account. For a 4 ml cell suspension, the volume doubles to 8 ml after 4 h of pulsing (5 h of starvation), decreasing the cell density by half.
11. Do not boil the cell lysates prior to immunoblotting since this causes cAR1 receptors to aggregate.
12. The primary antibody dilution can be reused multiple times due to the addition of NaN_3. The antibody should be stored at 4°C between uses. With repeated use, the signal from the antibody becomes weaker, and higher exposure times may be necessary during detection.

13. If the primary antibody yields a weak cAR1 signal and strong background signals, cAR1 expression can alternatively be analyzed using CHAPS insoluble floating fractions (CHIFFs) rather than whole-cell lysates (19). Spin down 5×10^6 cells at 360 rcf for 3–4 min, discard the supernatant, and resuspend the cell pellet in TEB (40 mM Tris–HCl, pH 8.0, 2 mM EDTA, and 50 mM NaCl in water). Centrifuge the cell suspension, discard the supernatant, and resuspend the pellet in 600 µl of ice-cold TEB supplemented with a protease inhibitor cocktail (Roche). Add 300 µl of 60 mg/ml CHAPS in TEB with protease inhibitors and incubate the cells on ice for 5 min. Spin down the cells at maximum speed for 15 min. Remove the supernatant and resuspend the pellet in 80 µl of 1× sample buffer (without boiling). Load 5–10 µl of the sample on a gel and proceed with steps 3–9 as described (see Subheading 3.3.2).
14. A rolled up Kimwipe can be placed in the tray with the membrane to absorb the Coomassie dye and speed up the destaining process.
15. Drops can also be dispensed by pipetting 1.5 µl of liquid with a Pipetman; however, this requires a significantly longer amount of time since the pipette needs to be refilled with each drop.
16. The chambered cover glass should be handled carefully as it is being prepared for microscopy, since abrupt movement may cause the drops to spread and/or move.
17. It is not recommended to develop more than two or three different cell lines at a time for use with the micropipette assay. Once developed for 5 h, cells can be used for a maximum of roughly 2 h (the amount of time necessary to analyze two to three cell lines with this assay) before they form tight aggregates that cannot easily be dissociated. To test more than three cell lines on the same day, the initiation of development can be staggered. Alternatively, developed cells can be placed on ice and used for the assay at a later time, although the cells should not be left on ice for more than a few hours and require approximately 15 min to recover at room temperature in the chambered cover glass before imaging.
18. Handle the femtotip gently to prevent cracks or breaks. After filling it with the cAMP solution, the tip of the micropipette should be examined under a microscope, using a 10× objective, for the presence of air bubbles. Any observed bubbles can be dislodged by gently flicking the upper plastic portion of the micropipette while its tip points downward. Additionally, before placing it in the chambered cover glass, the femtotip should be examined under a microscope for the presence of any blockages that could prevent consistent

cAMP flow. This can be done using the cleaning function of the microinjector. When the micropipette is cleaned, drops of cAMP should be seen coming from the tip.

19. Alternatively, a 1×10^{-6} M cAMP solution can be used to fill the micropipette. In this case, the compensation pressure should be increased to 60 hPa.
20. The cAMP should be added very carefully to avoid disturbing the cells or microscopy chamber, which could shift the focus of the image. Gently add the cAMP by holding the pipette tip above the well containing the cells, rather than submerging the pipette tip in the liquid. This ensures that the cAMP will not hit the cells directly with enough force to cause them to detach from the surface of the well.

Acknowledgments

The authors would like to thank Huaqing Cai for helpful comments and suggestions. KFS is supported by the American Heart Association. This work was supported by National Institutes of Health Grants GM34933 and GM28007 to PND.

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