# Regulation of Chemoattractant-mediated G protein Signaling

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BRIEF. The use of fluorescence microscopy to observe the localization of signaling molecules.

ABSTRACT. When the growth and division of cells becomes deregulated the results often lead to cancer. Studying the roles played by G-protein signaling molecules may allow us to understand the mechanisms involved in cell division and lead to the development of targeted drugs to control rates of cell replication that can lead to cancer. The regulation of cell division (cytokinesis) is frequently studied in the amoeboid protozoan *Dictyostelium discoideum.* Many of the same genes found in higher eukaryotes are homologous in *D. discoideum*. Preliminary data suggested cells were quiescent to external stimulation at the onset of cytokinesis. To further demonstrate this, cells were stimulated with the folic acid chemoattractant at various stages during cytokinesis and biosensors were observed to determine whether various signaling cascades are active. *D. discoideum* cells were imaged using fluorescence microscopy to observe the activity and localization of microtubules and the signaling molecules, Ras and PI(3,4,5)$P_3$, tagged with the green fluorescent protein. The cells were stimulated with folic acid at metaphase and after cytokinesis. PI(3,4,5)$P_3$ levels were unchanged. However, Ras was activated in response to chemoattractants, averaging an increased pixel intensity of 33%. Further studies will look at other signaling molecules to determine whether they are also activated.

INTRODUCTION.

Normal growth and division of cells in the human body are under tight regulation and are controlled by many signals which provide the stimulus for starting and stopping cell division. These factors are crucial to human health. When a problem occurs in this communication, cells continually divide and can eventually form a cancerous tumor. If they become metastatic, the tumors will start invading other tissue. This migration into other tissues often uses chemotaxis, a process in which cells sense chemical gradients and typically move towards the chemical source released from an organ or tissue [1]. Receptors coupled to G protein found along the cell membrane sense these chemicals and control this process. Chemotaxis, as well as cell division, can easily be observed in the model organism *Dictyostelium discoideum*, a microscopic amoeba that is commonly found in soil and feeds on bacteria [2].

Using fluorescence microscopy, it was found that the *D. discoideum* cells were unable to respond to outside chemical cues when in metaphase. At all other phases during cell division, the cells would respond when stimulated with folic acid, a chemoattractant active in growing cells in the vegetative cycle. This response was visualized as signaling molecules involved in the cells' functions became activated and changed locations within the cell [3]. These signaling molecules were genetically tagged with the green fluorescent protein (GFP) to identify their spatial location within the cell. When activated, the signaling molecule, in this case PH-GFP, which binds to a membrane lipid PI(3,4,5)$P_3$, moved from within the cell to the membrane periphery [4]. This exterior edge experienced a brightness that is visually more intense than the other parts of the cell [3].

The main goals of this research were to measure signaling responses at different stages of the G-protein signaling cascade and to determine at what point along the pathway the block in signaling was occurring. Determining the cause of the signaling inhibition could aid in the creation of drugs capable of preventing cellular migration and division. It may be possible to manipulate other cells so that they exhibit similar behavioral conditions to the unresponsive cells in metaphase. This could lead to the ability to control the unregulated growth and division of cells that results in cancer. Manipulations of molecules in the single-celled amoeba just may be the key to understanding how to stop cancerous cells from proliferating.

MATERIALS AND METHODS.

*Cell Lines.*

Transformations of the GFP-tagged PI(3,4,5)$P_3$ and Ras were performed to generate the various cell lines. The PH domain serves as a marker for PI(3,4,5)$P_3$, and RBD is a biomarker for the activated form of the small G-protein Ras [5].

*Cell Culture and Plating.*

The *Dictyostelium discoideum* cell lines used were cultured in HL-5 liquid media (Formedium) [5]. The cells were plated on SM agar (Formedium) along with the bacterial food source *Klebsiella aerogenes*. The cell mixture was incubated at room temperature for 18 to 20 hours. Cells were washed three times in 1 mL development buffer (DB) containing 5 mM $Na_2HPO_4$, 5 mM $KH_2PO_4$, 1 mM $CaCl_2$, and 2 mM MgCl2 to remove any remaining bacteria which may interfere with the fluorescent microcopy analysis [5]. The cells were then centrifuged at 2000 rpm and prepared for imaging.

*Microscopy.*

After completing three washes in DB, cells were placed in one-well chambers along with 2 mL DB. An Eppendorf micropipette was loaded with 7 µL of 10 µM folic acid. The micropipette was connected to an Eppendorf Femtojet micropump set at 150 hPa. Three time lapse series of individual images were taken. Once a cell was identified to be in metaphase based on its morphology, images were collected using a Cool Snap CCD camera in the MarianasTM Workstation every three seconds. After five frames the micropipette was brought down to a pre-set position in close proximity to the target cell in order to determine whether translocation of the biosensor to the membrane occurred. After another 15 frames the micropipette was brought up to a second position near the surface of the DB so that it would not become saturated with folic acid. A second video was started with images taken every 10 seconds until the cell completed division. At which point, a third video was taken with frames every three seconds for a total of twenty frames. Cells were again stimulated with folic acid after five frames to see whether a response occurred. Images were taken at 40X on a Zeiss Axiovert Marianas Workstation from Intelligent Imaging and Innovations fluorescent microscope. A GFP filter (excitation HQ480/20x, emission HQ510/20m) was used with an exposure of 500 milliseconds. The target goal was to acquire ten time lapse videos for each cell line.

*Data Analysis.*

Images were viewed using Slidebook (Intelligent Imaging Innovations, Inc., Denver, CO). It was determined whether the cells responded to the stimulation of the folic acid chemoattractant by observing whether there was a change in localization to the membrane. Responses were quantified by individually masking the cytosol and membrane of each dividing cell and control cell to calculate the average intensity both before and during stimulation. The masks were manually performed using Slidebook. A uniform width surrounding the edge of the cells was used to mask all cell membranes. The background area was also masked and subtracted out to normalize for variations in exposure time. The percent change in pixel intensity could then be calculated.

RESULTS.

To determine if the *D. discoideum* cells respond to the exogenous folic acid during different stages of cytokinesis, the localization of certain biomarkers were

observed. As previous data suggested, the PH-GFP cells in metaphase did not respond when stimulated with folic acid. When the *D. discoideum* cells in metaphase were first imaged, the PH domain had accumulated in the cytosol since plasma membrane levels of $PI(3,4,5)P_3$ dropped. When the metaphase cell was stimulated with folic acid, no response was seen (Figure 1). The PH domain was not recruited to the membrane. However, the surrounding control cells not in metaphase responded with the PH translocating to the membrane when stimulated with chemoattractant (Figure 1). At the point of stimulation, the edge of the control cell had a higher fluorescence signal that was visually brighter than that of the cytosol.

An upstream protein Ras was also viewed. Target cells in metaphase could easily be identified as they were round and had RBD-GFP accumulating in the cytosol. When RBD-GFP cells were stimulated, the biosensor was recruited to the membrane at all stages of cytokinesis. The response was visualized as the perimeter of the cell developed an intense brightness, stronger than that of the rest of the cell (Figure 2). The response disappeared when stimulation stopped, but was seen once again just as the two daughter cells resulting from cytokinesis were about to split. Intense green fluorescence could be visualized at the membrane (Figure 2). A strong response was seen both before and after cytokinesis.

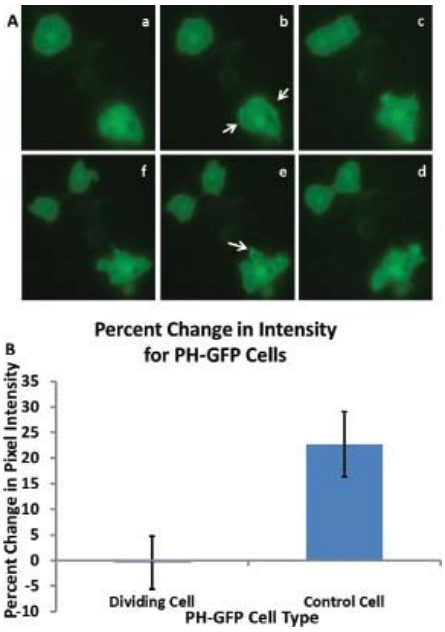


**Figure 1.** (**A**) Two cells expressing PH-GFP are being shown. The top left cell in each box starts out in metaphase (a) and progresses through cytokinesis. The progression through division can be seen in boxes a-f. The bottom right cell in each box is the positive control. The dividing cell does not respond when stimulated, but translocation of PH-GFP can be seen in the positive control (b and e). The arrows indicate where the response is seen at the membrane. (**B**) The average percent change in pixel intensity for the dividing cells and control cells are being compared. The p-value for this data set is 1.48E-06.

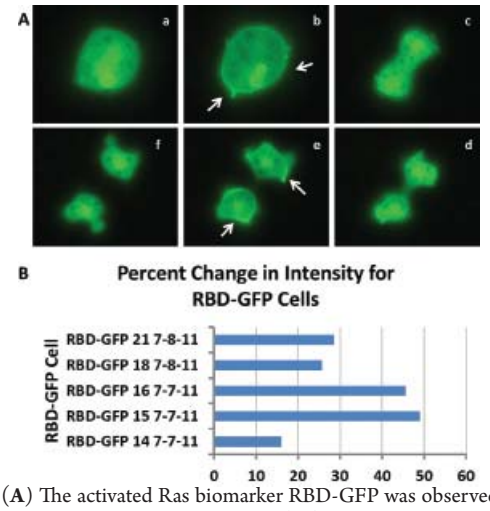


**Figure 2.** (**A**) The activated Ras biomarker RBD-GFP was observed as a single *D. discoideum* progressed through cytokinesis (a-f). Before stimulation, a rounded up cell was located (a). The cell was stimulated in metaphase (b) and at the completion of cytokinesis (e). It responded in both phases. The arrows indicate where the RBD-GFP localizing at the membrane occurred. (**B**) The range of percent intensity changes in RBD-GFP cells is displayed. The responses were quantified a frame before stimulation and at the point of greatest intensity.

DISCUSSION.

An experiment was devised to stimulate dividing cells with chemoattractants to investigate how the cells would respond as they became bipolar. No translocation was noted when metaphase cells expressing PH-GFP were stimulated (Figure 1). However, contrary to the original hypothesis, not all *D. discoideum* pathways are quiescent in metaphase. Cells tagged with RBD-GFP exhibited a strong response to folic acid even when in metaphase. Therefore, it is known that GPCR signaling is intact as well as the ability of the chemoattractants to activate the small G-protein Ras.

The PH domain binds to $PI(3,4,5)P_3$, whose levels rise when PI3K converts $PI(4,5)P_2$ to $PI(3,4,5)P_3$. Both Ras and the heterotrimeric G protein are at the plasma membrane, the site where activation initially occurs. After Ras is activated, it can then activate PI3K. Since Ras was activated at metaphase but $PI(3,4,5)P_3$ was not, this suggests a Ras independent pathway which turns off the ability of the cell to produce $PI(3,4,5)\ P_3$. From prior data it is known that PTEN-GFP intensely associates with the entire membrane of rounded up cells entering cytokinesis [3]. Too much PTEN on the membrane could possibly be the reason why the PH-GFP cells did not respond.

These results also gave us insight into a possible role of microtubules. The only time when the *D. discoideum* cells did not have microtubules was in metaphase. The microtubule network is disassembled at this stage [6]. Since parts of the signaling pathway were still able to respond while in metaphase, it can be inferred that microtubules are not essential for Ras signaling, but may play a role in regulating the spatial localization of PTEN, and ultimately the plasma membrane levels of $PI(3,4,5)P_3$.

These new findings further knowledge on the interaction between microtubules and various signaling molecules. In a study performed where cells expressing PH-GFP and PTEN-GFP were treated with the mitotic inhibitor nocodazole, little effect was seen on motility, but cells were delayed at mitosis. As cells rounded up at the onset of cytokinesis, PTEN and myosin II associated uniformly with the membrane, and $PI(3,4,5)P_3$ signaling was suppressed. Based on these prior experiments with nocodazole, it was suggested that events depending on the spindle microtubules control the spatially localized activities of PI3K and PTEN [3]. While this data is still only correlative, it is consistent with current findings, as the PH-GFP cells did not respond in the absence of microtubules. When the cells were at metaphase and microtubules were not

present, $PI(3,4,5)P_3$ signaling was also suppressed. This is consistent with other findings stating that the activation of Ras at the leading edge of chemotaxing cells is independent of the F-actin cytoskeleton, but PI3K localization is dependent on F-actin polymerization. It was demonstrated that without Ras, there is a loss of directional cell movement. In addition, it was shown that the translocation of PI3K to the plasma membrane requires the F-actin cytoskeleton and is not a part of the initial regulatory response [7].

The G-protein signaling cascade is an extremely complex system of pathways. Although there has been great strides made in progressing knowledge about these pathways, there is much that still remains undiscovered and unresolved. This current research is advancing the effort to determine the multitude of interactions that occur along the G-protein signaling cascade, in attempts to better understand the mechanisms that regulate cell mobility and division. Increased knowledge in this field may potentially lead to putative drug targets and alternative treatments to cancer, impacting the lives of millions affected by this disease.

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SUPPORTING INFORMATION.

**Figure S1.** G-Protein Signaling Cascade

**Figure S2.** Preliminary Data, Fluorescence Imaging of PH-GFP Cell

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