Examining the Role of Phosphorylation in Receptor Localization and Chemotaxis in *D. discoideum*

By

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DEDICATION

I want to dedicate my thesis to my amazing parents and my wonderful husband.

My parents have supported me, and pushed me to be everything I can. They spent thousands of dollars giving me a great education and everything I could need. They believed in me. I love you both and couldn't ask for better parents.

My husband has been my rock for the past two and a half years. When I thought I couldn't take any more of graduate school and wanted to leave, he was the light at the end of the tunnel. He spent hours watching me study and work. He loved me through my crazy moods, and all the nutso. I love you with all my heart and am so blessed to have you in my life.

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CHAPTER 1

cAR1 AND ITS SIGNALING PATHWAY

Background

Dictyostelium discoideum is a single-celled free-living social amoeba with a life cycle that relies on chemotaxis to find food and to survive adverse environmental conditions. *D. discoideum* is an excellent model system for studying fundamental processes in cell biology, such as chemotaxis, cytokinesis, phagocytosis, vesicle trafficking, cell motility, and signal transduction. It is also an ideal model for genetic studies because its genome sequence is available, it exists as an easily transformed haploid, and it is easily imaged using standard microscopy techniques (Manahan 2004).

Chemotaxis is a complex process where a cell is able to sense a chemical signal and migrate directionally in response to that chemoattractant gradient. Understanding chemotaxis in *D. discoideum* is important because it can be used as a model system for eukaryotic chemotaxis, which is implicated in many diseases. During directed cell migration receptors for cyclic AMP (cAMP), a chemoattractant, have been shown to be uniformally distributed on the plasma membrane and G-protein activation is thought to mirror ligand binding (Xiao et al., 1997, Janetopoulos, 2001). Yet many of the signaling molecules respond in a highly amplified way, which then induce a coordinated remodeling of the cytoskeleton to produce cellular movement, shown in Figure 1 (Iijima 2002, Iijima 2004, Keizer-Gunnink 2007). This directed migration can be broken down into three components: gradient sensing, motility, and polarity (Devreotes 2003, Janetopoulos 2008). Gradient sensing is the ability of a cell to detect an overall change of ligand occupancy across the periphery of a cell (Devreotes 2003). This detection then

leads to an internal polarized response that can result in motility. This is where a cell typically migrates towards higher concentrations of the chemoattractant by extending pseudopods or forming a leading edge in the proper direction. Polarity is the propensity of a cell to assume an asymmetric shape with a defined anterior and posterior, due to certain molecules and proteins being spatially restricted to these defined sectors (Janetopoulos 2003).

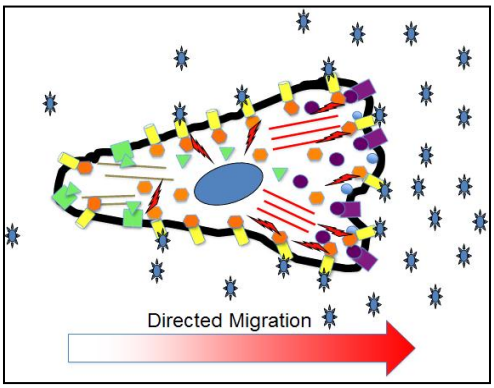


Figure 1 Chemotaxing Cell: When a cell is stimulated by ligand (blue stars) binding, a number of responses occur, including actin polymerization (red lines), myosin formation (green lines), $PI(3,4,5)P_3$ (lightning bolts) binding to PI3K (red hexagons), and PTEN (green triangles) localizing in the lagging edge.

Directed migration is shown to be a part of D. discoideum's developmental cycle, initiated by starvation. After a few hours, cells begin to secrete cyclic AMP (cAMP). These propagating waves of cAMP occur every 5-6 minutes, and increase in amplitude over the next several hours. They regulate the aggregation of cells into mounds. During the next 12-16 hours, cells within the mound differentiate and migrate to specified locations in the fruiting body, including the spore sac. These spores can be dispersed to nutrient-rich habitats, and then germinate and resume proliferation as individual amoebae (Kim 1997).

The oscillations of cAMP described above play at least two critical roles: they upregulate numerous genes required for the developmental phase of the *D. discoideum* life cycle, and they set up the gradients that are critical for chemotaxis during mound formation. The cAMP receptor 1 (cAR1) is a serpentine receptor that, along with its heterotrimeric alpha subunit, $G\alpha_2$, are up-regulated in the first few hours after starvation and are fully expressed by the mound formation stage (Hereld 1994). This G-protein coupled receptor

(GPCR) are structurally characterized by a bundle of seven transmembrane alpha helices interconnected by alternating extracellular and cytoplasmic loops. Most GPCR ligands are thought to bind transmembrane or extracellular receptor domains, while the G-proteins interact with the cytoplasmic loops. cAR1 contains four extracellular domains, three intracellular loops, and an intracellular C-terminal domain (Hereld 1994). The third intracellular loop interacts with the heterotrimeric subunit, comprised of the $G\alpha_2$ and $G\beta\gamma$ subunits, of which the carboxyl terminus of the $G\alpha_2$ subunit is the primary determinant of receptor coupling, seen in Figure 2. When the chemoattractant, cAMP, binds to cAR1, it triggers the complete dissociation of G-protein into $G\alpha_2$ and $G\beta\gamma$ subunits (Elzie et al. 2009). cAR1 mediates the activation of $G\alpha_2$ by functioning as a guanine nucleotide exchange factor (GEF). Thus, cAMP binds to the receptor, and conformational changes within the receptor internalize the response and activate further downstream signals to mediate actin polymerization, calcium uptake, signaling, chemotaxis, and differentiation. The activation of heterotrimeric G-proteins by cAR1 and the sequence of events following this activation are highly analogous to those also described in mammalian cells (Zhang 2005, Oldham 2008). These similarities include activation of Ras, PI3K, and PKB. Free $G\beta\gamma$ activates Ras, a small G protein, which in turn triggers the activity of phosphoinositide 3-kinase (PI3K), an enzyme that converts $PI(3,4)P_2$ to $PI(3,4,5)P_3$. $PI(3,4,5)P_3$'s can serve as a binding site for proteins with Plesktrin Homology (PH) domains, such as the cytosolic regulator of adenylyl cyclase (CRAC) and protein kinase B (Akt/PKB). The synthesis of $PI(3,4,5)P_3$ rapidly recruits these proteins to the cell membrane, which in turn play a role in regulating the actin cytoskeleton. Activation of cAR1 also regulates membrane association of 3-phosphoinositide phosphatase (PTEN), which converts $PI(3,4,5)P_3$ to $PI(3,4)P_2$.

In addition to its role in signal transduction, cAR1 is uniformly distributed across the surface of a cell during stimulation (Hereld 1994). The binding of the ligand, cAMP, during stimulation is required for phosphorylation of thirteen essential residues in the c-terminal tail of cAR1 (Caterina 1995). Phosphorylation of five of these residues accounts for two-thirds of the total phosphorylation, and that is attributed to five serine residues:

S299, S302, S303, S304, and S308 (Caterina 1995). Analysis of mutants lacking combinations of these serines revealed that either S303 or S304 is essential for functionality; mutants lacking both serines are defective in all responses (Caterina 1995). A decrease in ligand binding is attributed to reduction in binding affinity or internalization of the receptors, the latter of which has not been determined to date. Deletion of these five residues is sufficient for a five-fold reduction in affinity of cAR1 for cAMP (Caterina 1995). A mutant in which all five serines in the cluster were substituted with alanines exhibits reduced cAMP-stimulated phosphorylation and failed to undergo the electrophoretic mobility shift (Caterina 1995).

Many models have been proposed to explain the transient responses of cells to uniform stimulation and the prolonged responses of cells in a gradient (Devreotes and Janetopoulos, 2003). One model, the local excitation, global inhibition (LEGI) model, predicts that cAMP-occupied cAR1 sends out both an excitatory signal that activates downstream signaling effectors molecules and an inhibitory signal that terminates the response. The balance between excitation and inhibition in a gradient leads to a localized response. At a given concentration, the model predicts that as the fraction of the cell stimulated increases, the concentration of the inhibitor should also increase, leading to a smaller difference between excitation and inhibition, and consequently, a smaller response (Janetopoulos 2004).

The removal of cAMP initiates a process of de-adaptation. Cells can respond again to cAMP in about 3-4 minutes (Caterina 1995). Adaptation, a rapid reduction in agonist-induced effector activation, has been attributed to the uncoupling of receptor from G-protein (Caterina 1995). This uncoupling is proposed to result from agonist-induced receptor phosphorylation (Caterina 1995). In mammalian systems, the second event is the subsequent association of arrestin, which appears to obstruct further receptor-G-protein interaction. The third event in *D. discoideum* is an agonist-induced desensitization process that causes a rapid reduction in the apparent number of surface binding sites (Xiao 1997). Desensitization is a series of processes that prevent continuous activation of the cell during prolonged exposure to agonist, thus protecting a cell and organism from overstimulation (Xiao 1997, Ferguson 2007, Nguyen 2011,

Adams 2011). In some instances, this loss of ligand binding, often referred to as sequestration, has been attributed to receptor endocytosis or internalization (Caterina 1995). Previous studies have shown that chemoattractant receptors remain uniformly distributed on the surface of cells that have been polarized by chemotactic gradients and also in cells that have been desensitized by persistent treatment with chemoattractant (Xiao 1997).

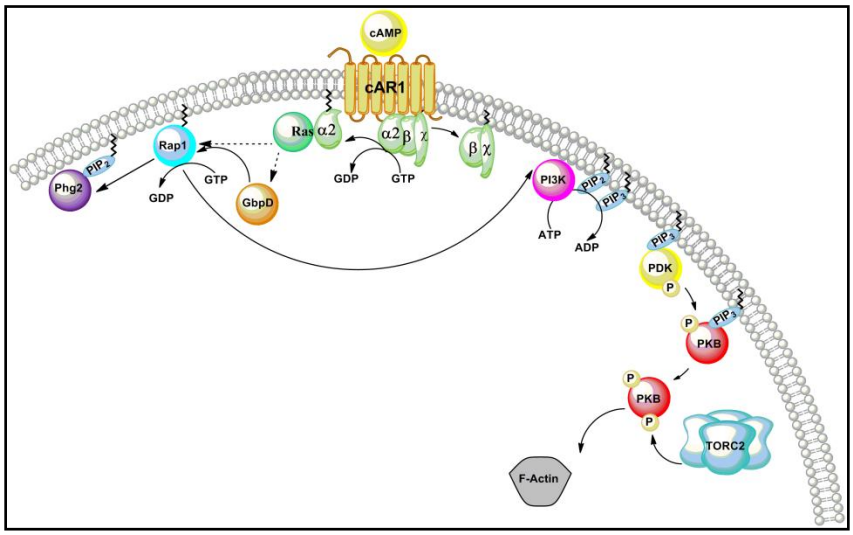


Figure 2 Signaling Cascade: When the chemoattractant, cAMP, binds to cAR1, it triggers dissociation of G-protein into $G\alpha_2$ and $G\beta\gamma$ subunits. Free $G\beta\gamma$ activates Ras, a small G protein, which in turn activates phosphoinositide 3-kinase (PI3K), an enzyme that converts $PI(3,4)P_2$ to $PI(3,4,5)P_3$. $PI(3,4,5)P_3$'s serve as binding sites for the proteins with PH domains, such as cytosolic regulator of adenylyl cyclase (CRAC) and protein kinase B (Akt/PKB), and once generated, they recruit these proteins to the cell membrane. These proteins play a role in regulating the actin cytoskeleton. Activation of cAR1 also regulates membrane association of 3-phosphoinositide phosphatase (PTEN), which converts $PI(3,4,5)P_3$ to $PI(3,4)P_2$.

PI3K has been implicated in the response of actin polymerization during polarity and chemotaxis (Korholt 2010). When levels of $PI(3,4,5)P_3$ rise, Akt/PKBA is recruited to the plasma membrane, is activated and phosphorylated (Korholt 2010). In migrating cells, these phosphorylation events are restricted to the cell's leading edge (Korholt 2010). Thus, PKB and PI3K are essential for proper cell polarization and movement in a chemoattractant gradient. Polarity, the propensity of a cell to assume an asymmetric shape with a defined anterior and posterior, is regulated by factors activated after the binding of cAMP to cAR1 (Devreotes and Janetopoulos 2003). Even in a highly polarized cell, a sufficiently steep gradient applied to the side or back can break the

polarity and create a new front (Devreotes and Janetopoulos 2003). To help produce an anterior and posterior of the cell, Pleckstrin-homology (PH) domain containing proteins, such as CRAC and PKB, are recruited to $PI(3,4,5)P_3$ on the plasma membrane, thus localizing asymmetrically to other proteins. When cells are placed in a gradient, these proteins bind to $PI(3,4,5)P_3$, synthesized at the leading edge. $PI(3,4,5)P_3$ is a product of PI3K activation, and it's levels are regulated by the tumor suppressor PTEN, a phosphoinositide 3' specific phosphatase that dephosphorylates $PI(3,4,5)P_3$ to $PI(4,5)P_2$ (Iijima 2002). In resting cells, PTEN is localized to the plasma membrane and uniformly distributed around the cell. In response to global stimulation, PTEN transiently dissociates from the plasma membrane with kinetics that are inversely proportional to those of PI3K plasma membrane association (Iijima 2002). In a cAMP gradient, PTEN relocalizes to the sides and the back of the cell, and likely helps specify the rear of the cell. When a gradient is moved, polarized cells will adjust their leading and lagging edges accordingly; polarized cells that are given a uniform stimulus of cAMP will respond preferentially at the leading edge. This is referred to as polarized sensitivity (Janetopoulos 2003).

When cAR1 was genetically tagged with GFP, it was uniformly localized on the plasma membrane during chemotaxis and also following prolonged uniform stimulus (Xiao 1997). This data showed that cAR1 did not apparently undergo a significant redistribution during phosphorylation. In addition only 15-25% of the receptor was sequestered, with a reduced affinity for ligand (Xiao 1997). De-adaptation appears to occur at the plasma membrane, thus can be detected using single molecule microscopy, which have provided some insight to receptor/ligand interactions. Using Cy3-cAMP, cAR1 binding sites were found to be uniformly distributed and diffused rapidly on the plane of the membrane (Hereld 1995, Ueda 2001). The on and off rates were faster at the front of the polarized cells. It is likely that this resulted in a higher frequency of G-protein association and dissociation (Ueda 2001). Also, agonist-induced phosphorylation had no effect on this frequency or the distribution of the receptor (Ueda 2001). These results suggest that receptor occupancy and G-protein activation parallel the external gradient

and there is little amplification of the gradient in these early steps (Janetopoulos 2001, Ueda 2001).

Although the signaling pathway is fairly well characterized, there are many details missing when it comes to understanding the regulation of receptor desensitization and adaptation of the response, which are two critical components of gradient sensing. Cells are able to sense very shallow gradients over several orders of magnitude of chemoattractant; it is still unclear how they manage this remarkable task. Furthermore, we know that the signaling cascade needs to be intact not only for motility, but for polarized morphology and sensitivity as well. It is of fundamental interest to determine the role of receptor phosphorylation in influencing these cellular events.

Phosphorylation of the C-terminus of cAR1 is thought to provide a role in the ability of cells to adapt to cAMP and respond to spatial gradients that span several orders of magnitude. It has been demonstrated that phosphorylation of cAR1 residues 299, 302, 303, and 304 regulate the affinity of the receptor for cAMP (Caterina 1995). I hypothesized that the phosphorylation status of the receptors allow them to respond to a wide dynamic range of cAMP concentrations and helps regulate the adaptation and de-adaptation of cellular responses to external cAMP. My work suggests that the phosphorylation state of the receptor contributes to the polarized redistribution of receptors and helps the cells chemotax in gradients with dramatically different mean concentrations. Understanding how these receptors polarize is of fundamental importance since they are critical for cAMP-mediated responses and the ability of a cell to sense concentration gradients of vastly different slopes and mean concentrations.

CHAPTER 2

LOCALIZATION OF cAR1 DURING CHEMOTAXIS

Introduction

Chemotaxis is the physiological phenomenon of cells migrating directionally toward or away from chemical cues. Chemotaxis is involved in many pathological diseases in humans such as cancer metastasis, autoimmune diseases, and developmental defects. *Dictyostelium discoideum* is a model system for studying eukaryotic chemotaxis. *D. discoideum* cells exposed to a gradient of cAMP will move toward that source of cAMP. The major serpentine receptor for cAMP, cAR1, is coupled to the heterotrimeric G-proteins α_2, β, and γ, which activate a host of downstream effectors including PKB, PI3K, and Ras to regulate cell shape changes, polarity, and motility. Phosphorylation of cAR1 has been shown to regulate the receptor's affinity for cAMP. Specifically, phosphorylation of cAR1 at serine residues 299, 302, 303, 304, and 308 results in a 5-fold decrease in cAMP *in vitro*. Furthermore, residues 303 and 304 are the primary serine residues responsible for the loss of ligand binding, suggesting that phosphorylation of cAR1 and its subsequent decreased affinity for cAMP may be important for receptor sensitivity and could potentially contribute to receptor adaptation for ligand. It is hypothesized that phosphorylation of cAR1 residues 299, 302, 303, and 304 regulate the affinity of the receptor for cAMP, and thus allow the cell to be able respond to a wide dynamic range of cAMP concentrations, which regulates adaptation and de-adaptation of cellular responses to external cAMP. It is expected that chemotaxis will be significantly decreased in the mutants compared to wildtype cells.

Methods

Media and buffers:

HL-5 was purchased from Formedium. HL-5 media consists of 22 grams of HL-5 powder, 10 grams of dextrose and 1 Liter of double distilled H2O. Developmental Buffer (DB) consists of 5mM Na_2HPO_4, 5mM KH_2PO_4, 1mM $CaCl_2$, and 2mM $MgCl_2$.

Strains used:

Dictyostelium discoideum wild type strain expressing cAR1-GFP and mutants expressing cAR1-GFP A4 (S299A, S302A, S303A, S304A) or cAR1-GFP E4 (S299E, S302E, S303E, S304E) were used for cAMP chemotaxis. These mutants were expressed in a wildtype AX2 background, as well as a cAR1-/3- background. These strains confer G418 resistance.

The original strains were obtained from the Devreotes laboratory at Johns Hopkins, including cAR1/3.1 and cAR1/3.2. cAR1.1 is the A4, E4, and WT in car1/3.1 background. The cAR1 gene contains a BgII insertion. cAR1.2 is the A4, E4, and WT in car1/3.1 background. The cAR1 gene does not contain BgII insertion. cAR1.3 is the E4 and WT in car1/3.2 background. The cAR1 gene does not contain BgII insertion.

*It is to be noted that cAR1.3-A4-GFP cells were never achieved in rounds of transformations when deleting the BglII site mutation. This is presumably due to the interaction of the BglII site mutation and the A4 mutations in the c-terminal tail. The wildtype cAR1-GFP was transformed into cAR1.1, AX2 (wildtype background), and cAR1.3.

cAMP preparation:

10mM stock of cAMP (Sigma) solution was made in double distilled H_2O. For cAMP development a 2.5 μM working solution was made in DB buffer. For cAMP chemotaxis, a 100 nM, 10 μM, and 10 mM working solution was made in DB buffer.

cAMP development:

50 mL of $2x10^6$ cells per ml were centrifuged at 1400 rpm and subsequently washed twice times with DB buffer. The pellet was resuspended in 5 mLs of DB buffer resulting in 2×10^7 cells per mL. The 5 mL resuspension was transferred to a 150 mL flask and was shaken at 110 rpms for 1hr. After the initial 1hr shake, the cells were pulsed with approximately 100 μL of the 2.5 μM cAMP every 6 minutes for 5 hrs. After the 6hr development, the cells were treated with 5mM caffeine (only for biochemical studies) and shaken for 30 minutes to basalate the cells or the cells were immediately used for assays.

Imaging:

All images were acquired on a Marianis Workstation equipped with a Cool Snap CCD camera and an Extended QE, high-speed cooled CCD camera (Cascade 512 II). The microscope used was an inverted, wide-field epifluorescence microscope (Zeiss Axio Observer Z1). Images were acquired by SlideBook software (Intelligent Imaging Innovations).

Cell tracking:

In order to visualize the migration of cells and record the specifics of chemotactic response; a program was written with LabVIEW™ software. Time-lapse image data from Slidebook was exported to .avi movie format. The movie files were loaded in the cell-tracking program. The user can view the image data in a window, play the file or examine individual frames. Tracking of cells uses the machine vision algorithms from LabVIEW™ Vision Development Package. The user selects the cell to track with a mouse, and the software records the x,y pixel coordinates of the centroid of intensity in the image for the fluorescent cell. Once the x,y path of the cell is known and the source of the chemoattractant is identified the chemotactic response can be quantified. The measured responses are the velocity of travel, the direction of travel (chemotactic index), and the persistence. The velocity is simply the distance between x,y pairs of the cell path divided by the time between points. Velocity can be measured point-to-point or

integrated over several time steps. Chemotactic index (CI) is the cosine of the angle formed by the travel vector and the vector from the cell to the source. The travel vector is determined by the x,y track of the cell. The travel vector can be taken at each frame increment or integrated over several frames where CI (n) = cos (angle S X_n X_{n+m}). CI is the chemotactic index of the cell at time n, S is the point source of the chemoattractant, X_n is cell's position at the start point of the travel vector, and X_{n+m} is the cell's position at the end point of the travel vector after the integration time increment m. It is useful to calculate the velocity and chemotactic index over short time scales corresponding to the movement of one cell length. For these time scales the chemotactic persistence, defined as the length of cell travel path divided by the change in distance to the source, is equal to the chemotactic index. The calculation of persistence is more useful over long time scales.

Gene Expression Assay

After treatment of caffeine, developed cells were centrifuged for 5 minutes at 500 x g at 4^{o}C, and the supernatant was removed. This was done twice with 30mL of ice cold DB buffer. The cells were resuspended at 2 x 10^7 cells/mL in DB and kept on ice. 0.5-mL of competent cells on ice at a density of 2 × 10^7 cells/mL were transferred to a 5-mL flask shaking at 110 rpm. Within 2 minutes, 100 uM of cAMP were added to a final concentration of 1uM. A Western Blot was performed. This was done by transferring 50uL of cells to microcentrifuge tubes containing 17 uL of 4 × SDS sample buffer and heated at 95°C for 5 minutes. Wells were loaded in precast gels with 2.5 uL of sample (4 × 10^4 cells). Gels were run at 200 V for 90 minutes. Proteins were transferred to a nitrocellulose membrane at 30 V for 90 minutes. After the transfer, the nitrocellulose membrane was rinsed with TBS twice. The membrane was incubated in 10 mL of blocking buffer for 1 hour at room temperature while shaking. The blocking buffer was discarded and the membrane was rinsed with TBS twice. The membrane was incubated with a primary antibody 1:1000 at 4°C overnight. The primary antibody was removed, and the membrane was rinsed with TBS twice, and further washed with TBST for 10 min, thrice at room temperature. The membrane was incubated with IR dye 680 conjugated secondary antibody (Li-Cor) 1:10,000 for 1 hour at room temperature. The

secondary antibody was removed, and the membrane was rinsed with TBST thrice at room temperature. It was washed quickly with TBS twice and scanned in the Odyssey IR dye detection system. PKB substrate phosphorylation assays were also performed in the same way using a PKB substrate phosphorylation specific primary antibody.

PCR Mutagenesis

PCR (Polymerase chain reaction) was performed using Pfu polymerase, 10 mM dNTP, template, and primers. The PCR was performed for 15 cycles, with a 10 minute extension time. After PCR, the product was restriction digested for three hours at 37^{o}C. The DNA was precipitated and ligated at 18^{o}C overnight. A bacterial transformation was performed using competent cells. The cells were thawed on ice, mixed with the ligated DNA, and heat shocked at 42^{o}C for one minute. After allowing 1 hour for growth during shaking at 37^{o}C, the cells were plated on an LB/Ampicillin plate. Colonies were picked from each plate, grown overnight at 37^{o}C, and precipitation for isolation of the finished plasmid. This was performed multiple times to obtain a ДBglII construct for WT, A4, and E4. This plasmid was transformed into fresh *D. discoideum* cells.

DB agar/SM plates

Cells were developed as discussed above. These cells were plated on 1.5% agarose at 1:1 and 1:10 ratio to detect aggregation defects. In short, 1mL of cells were plated, the cells were allowed to settle and adhere to the agar for 10 minutes, and the liquid was subsequently removed.

Latrunculin A treatment

During chemotaxis assays, cells were treated with Latrunculin A to prevent actin polymerization. The cells were suspended in 1.8 mL of DB buffer in a chamber slide. 1 uL of Latrunculin A was added to 199 uL of DB buffer, final concentration of 50 uM. This was added to the chamber slide during chemotaxis to achieve a final concentration of 5 uM drug treatment.

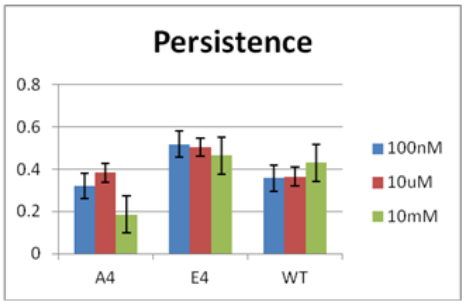
Subcloning

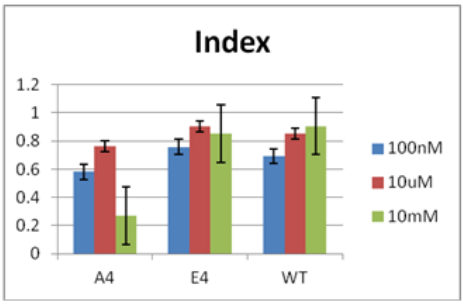
PCR subcloning was performed using Pfu polymerase, 10 mM dNTP, template, and both a forward and reverse primer. The PCR was performed for 35 cycles, with a 10 minute extension time. The PCR products were run out using gel electrophoresis. The bands were excised and digested using the Qiagen Gel Extraction Kit. After PCR, the product was restriction digested for overnight at 37^{o}C. The DNA samples were run out using gel electrophoresis, and the bands excised and isolated. The DNA was ligated at 18^{o}C overnight. A bacterial transformation was performed using competent cells. The cells were thawed on ice, mixed with the ligated DNA, and heat shocked at 42^{o}C for one minute. After allowing 1 hour for growth during shaking at 37^{o}C, the cells were plated on an LB/Ampicillin plate. Colonies were picked from each plate, grown overnight at 37^{o}C, and precipitation of the finished plasmid. This plasmid was transformed into fresh *D. discoideum* cells.

Results

Chemotaxis, Localization, and Latrunculin Treatment Round 1

The experiments presented here have been designed to characterize the cAR1:A4 and cAR1:E4 mutations and their effect on the cell's physiology. In the first cAR1/3- double null background, denoted as "cAR1.1.", the phospho-mimic, E4, has a chemotactic defect at high (10 mM) and low (100 nM) cAMP concentrations. Looking solely at chemotactic index, the cAR1.1:E4 cells travel towards the needle at 37% efficiency at 100 nM, 82% efficiency at 10 uM, and 77% efficiency at 10 mM. In the cAR1.1 knockout, the phospho-null, A4, has a chemotactic defect at all concentrations, with only about a 13% chemotactic efficiency at high and low concentrations, and 35% efficiency at 10 uM. Wild type cAR1-GFP expressed in cAR1.1 cells chemotax at approximately 89% efficiency at all concentrations. The speed of all of the mutants is comparable to wild type cells at about 7-10 microns/per minute. These results are seen in Figure 3, in graph form and in Table 1, a quantitative view. When treated with Latrunculin A, the cAR1.1:E4:PH-RFP cells respond to uniform cAMP stimulation; thus, the PH-RFP translocated to the plasma membrane, however, a crescent is not formed in the direction of the micropipette. Also, there is very little redistribution of the receptor with Latrunculin treatment. The cAR1.1:A4:PH-RFP cells do not respond to uniform stimulation, and a translocation event is not seen. cAR1.1:A4 cells expressing GFP tagged mutant receptors were treated with Latrunculin A. The A4-receptors redistributed to the entire cell, including the cytosolic compartments (data not shown). In very preliminary studies, cAR1.1:E4 cells treated with Latrunculin A do not appear to redistribute the E4 receptorSs; even in the absence of an actin cytoskeleton, intensity in the former lagging edge remains, with receptors in vesicles. All data was performed in the absence of caffeine, and confirmed.





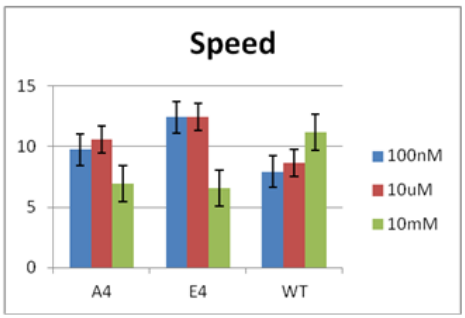


Table 1: Chemotactic Parameters

100nM	Persistence	Speed	Index
A4	0.320576	9.092319	0.125513*
E4	0.531929*	12.19629*	0.374478*
WT	0.351363	7.582161	0.220289
10uM			
A4	0.341042	9.73579	0.248937*
E4	0.491291*	12.95002	0.829579
WT	0.336572	8.618009	0.851918
10mM			
A4	0.175428	7.93524*	0.136295*
E4	0.458486	6.485728*	0.772107*
WT	0.429869	11.20569	0.906269

Figure 3 (A, B, C): Chemotaxis in cAR1.1 mutants with BglII site: All data was performed in the absence of caffeine, and confirmed. The concentration noted (100 nM, 10 uM, and 10 mM) is the concentration of cAMP in the needle during the assay. Persistence, speed, and index are defined in the Methods section.

The cAR1.1:A4-GFP and cAR1.1:E4-GFP, displayed a differential localization when imaged in polarized cells in a gradient (Figure 4). cAR1.1:E4, which is a phospho-mimic and should act as a desensitized receptor was found on the sides and lagging edge of the cell. cAR1.1:A4, should act as a phospho-null and be predicted to be a hyper-sensitive receptor, was found in the newly formed pseudopodia of a motile cell. This particular construct has given us problems, and these results need to be repeated again. We are hoping to get this construct without the BglII insertion to again test the distribution of the phosphonull receptor in migrating cells. Also, in a uniform stimulus, the mutant cells still maintained their differential localization (data not shown).

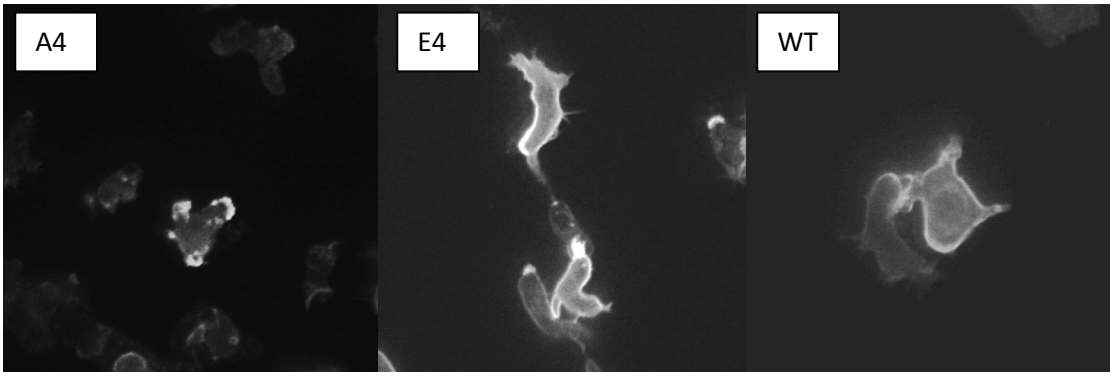


Figure 4: cAR1.1-GFP A4, cAR1.1-GFP E4, and cAR1.1-GFP WT Localization shown here uninduced.

Next, I decided to look at the time course of cellular events in response to a uniform stimulus of cAMP in the wildtype and mutant receptors. PKB substrate phosphorylation can be measure by Western blot analysis and is found downstream of major effectors, including heterotrimeric G-proteins, Ras, PI3K, and PIP3. Using PKB substrate phosphorylation, the signaling pathway is seen to be functioning, albeit at different intensities (Figure 5). The WT cells were not tested due to growth issues; however, it is postulated that they will saturate at high concentrations, similar to E4 cells. The E4 cells show a higher basal PKB phosphorylation level than A4, and seem to saturate at a higher concentration of 100 uM. The A4 cells terminate their response quicker at higher concentrations (above 10 uM), because they seem to saturate at much lower concentrations, and the response decreases with increasing concentrations of cAMP. This would lead us to test other downstream outputs and also use a time course to see if the kinetics are different for the mutants, compared to wildtype. Both of these mutants activate PKB, thus demonstrating that the signaling pathway regulating Ras activation, PI3K, and PI(3,4,5)P_3 signaling are functional.

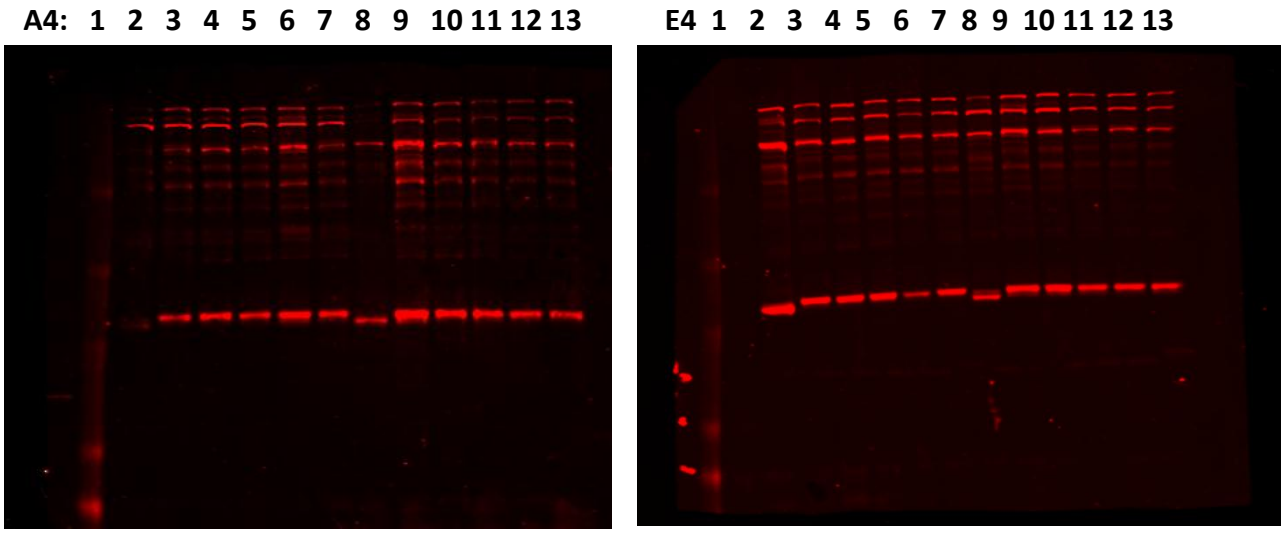


Figure 5: PKB Substrate Phosphorylation: The lanes are as follows: 1: Ladder, 2: Uninduced, 3: 10uM cAMP (no caffeine), 4: 20uM cAMP, 5: 40uM cAMP, 6: 60uM cAMP, 7: 100uM cAMP, 8: Uninduced, 9: 10uM cAMP (no caffeine), 10: 20uM cAMP, 11: 40uM cAMP, 12: 60uM cAMP, 13: 100uM cAMP; this is the same for both gels.

Chemotaxis Round 2: All mutations of cAR1 expressed in cAR1/3.1 and designated as the cAR1.2 cell line

In the second cAR1/3- set of experiments, we used cells denoted as "cAR1.2." The phospho-mimic, E4, was not tested due to mutagenesis issues. First, cells were tested for expression of the cAR1-GFP plasmid. These results are seen in Figure 6.

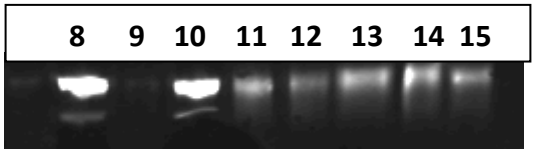


Figure 6: Gene Expression Assay: The lanes are as follows: 8: Positive Control, 9: Positive Control 2, 10: Blank, 11: cAR1.2-WT-GFP (30s), 12: cAR1.2-WT-GFP (60s), 13: cAR1.2-WT-GFP (90s), 14: cAR1.2-WT-GFP (120s), 15: cAR1.2-WT-GFP (uninduced).

The WT-cAR1.2-GFP showed very good chemotaxis efficiency >90% at both concentrations. The speed is comparative to wildtype (AX2) cells (approximately 10 microns/sec). The persistence is also comparable to wildtype cells at approximately 54-62%. These results show that the WT cAR1 fused to GFP is fully functional and rescues the cAR1.2 nulls.

The WT-cAR1.2-GFP construct expressed in a wildtype background, however, shows a chemotactic deficiency at 10 mM, where the index and persistence remains comparable to wildtype, but the speed is deficient. At 10 uM, the cells chemotax at an efficiency of 96%, 65% persistence, and 10 microns/sec. At 10 mM, the cells chemotax at 89% efficiency, 61% persistence, and 7 microns/sec. The reason for that deficiency could be that there are too many receptors due to the overexpression of the cAR1 gene. Developmental delays have been reported before when cAR1 is overexpressed, as is the case here.

The A4-cAR1.2-GFP construct showed very good chemotaxis efficiency >85% at all concentrations. The speed is comparative to wildtype (AX2), if not better than wildtype, at approximately 10.4-12.8 microns/sec. The persistence is slightly lower than wildtype as the persistence decreases as concentrations increase. The speed shows a similar profile of decreasing as concentration increases, though the variability is smaller. These results show a full rescue of cAR1 in the presence of cAMP.

The A4-cAR1.2-GFP- expressed in a wildtype background shows a chemotactic deficiency at 10 uM, where the index and persistence remains comparable to wildtype, but the speed is deficient, showing approximately 9 microns/sec. At 10 uM, the cells chemotax at impeccable efficiency of 96%, 60% persistence, and 9 microns/sec. At 10 mM, the cells chemotax at 89% efficiency, 62% persistence, and 11.6 microns/sec. The reason for the deficiency at lower concentration could be a saturation of receptor due to the overexpression of cAR1. Also, the two AX2 cell lines show a very similar profile, with high efficiency, persistence, and comparative speed, just as the cAR1.2 nulls show a similar profile. All of these results are seen in Figure 7 and Table 2 .

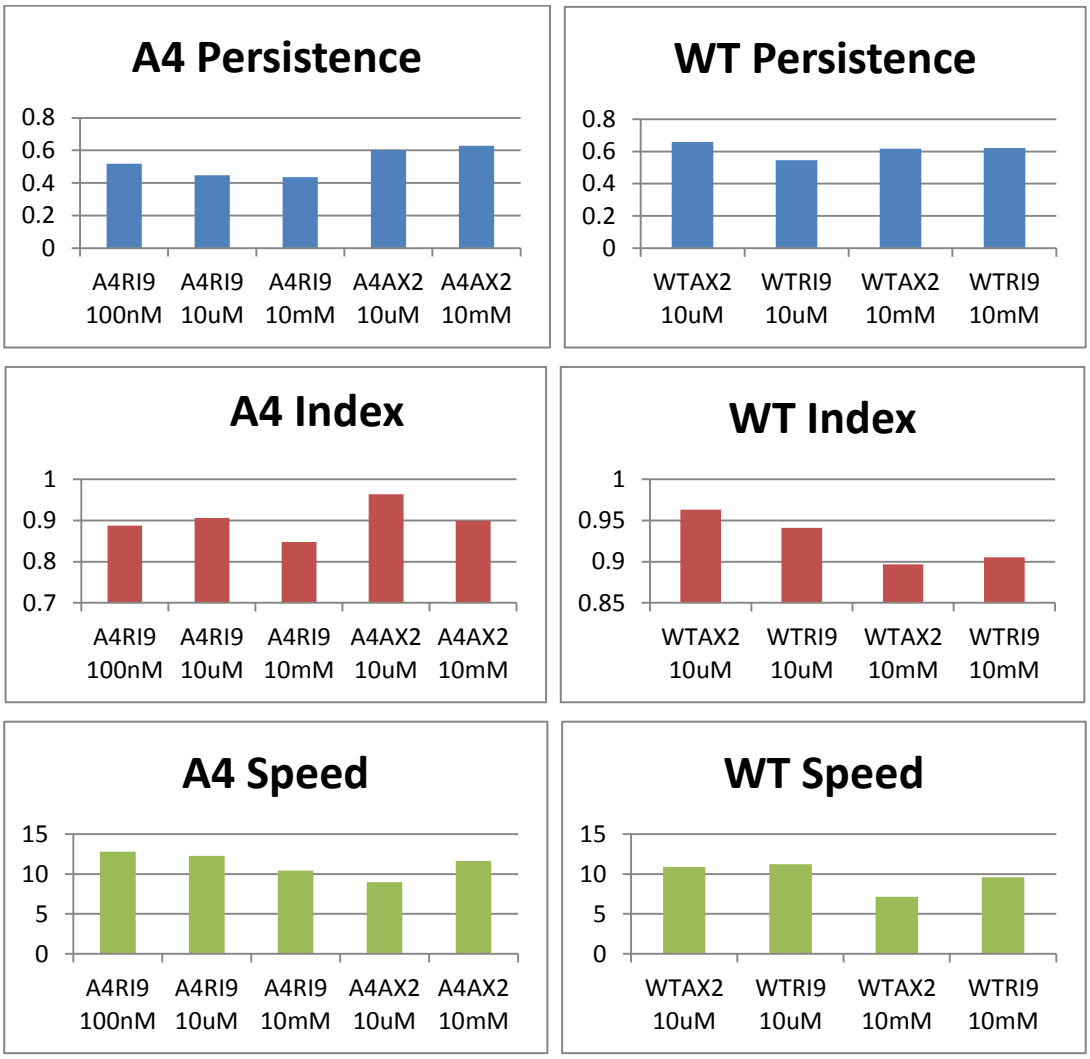


Figure 7: Chemotaxis without BglII site in cAR1.2: Both wildtype cAR1.3-GFP receptors and cAR1.3-A4-GFP were assayed for chemotactic parameters. Persistence, index and speed are defined in the Methods section.

Table 2: Chemotactic Parameters II

Cell	cAMP Concentration	Persistence	Index	Speed
cAR1.2-WT-GFP	10uM	0.545352	0.940805	10.2456
	10mM	0.620904	0.905291	9.58083
A4AX2	10uM	0.602392	0.963527	8.996255
	10mM	0.627433	0.898536	11.61405
cAR1.2-A4-GFP	100nM	0.517856	0.887494	12.7976
	10uM	0.446887	0.905853	12.26774
	10mM	0.435033	0.847362	10.44162
WTAX2	10uM	0.659481	0.962839	10.08964
	10mM	0.616906	0.896767	7.148719

Chemotaxis, Localization and Latrunculin Treatment Round 3: All constructs expressed in new cAR1/3- double null designated "cAR1.3"

The WT-cAR1.3-GFP cells were tested for chemotactic efficiency and in other experiments, the role of the cytoskeleton in redistributing the receptors was tested by inhibiting the actin cytoskeleton using Latrunculin drug treatment. The cells chemotax at a high efficiency (see Figure 8), and when treated with Latrunculin, the cells rounded up and the receptor did not redistribute. Also, the receptor in these cells is distributed uniformly, with a subtle difference in intensity in the back of the cells. The star is the location of the needle.

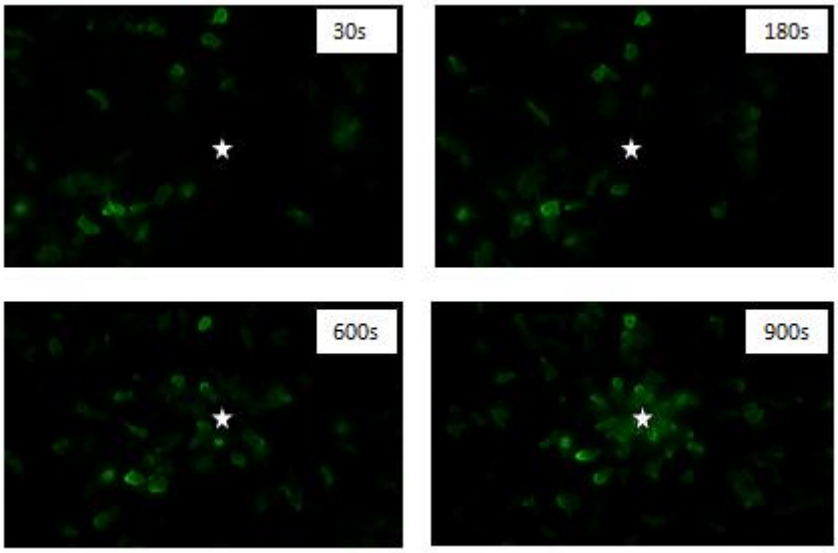


Figure 8: WT-cAR1.3-GFP Cells During Chemotaxis: The images reflect the cells' positions at 30s, 180s, 600s, and 900s.

The E4-cAR1.3-GFP cells were tested for chemotactic efficiency. The cells chemotax at a high efficiency, and when treated with Latrunculin, the cells rounded up and the receptor did not redistribute. Also, the receptor in these cells shows a localization of intensity in the back of the cells, with a gradient up the sides of the very elongated cells, seen in Figure 9.

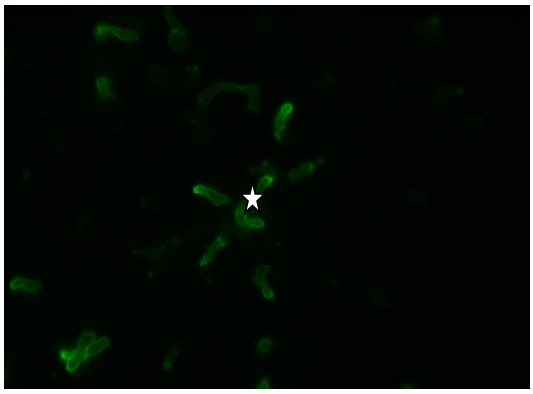


Figure 9: E4-cAR1.3-GFP Localization during Chemotaxis taken at 1200 seconds.

DB Agar and SM Bacterial Plates

The DB Agar plates were used to show the ability of the different receptors to rescue development and aggregation. All cell lines were tested, and the results reported in Figure 10.

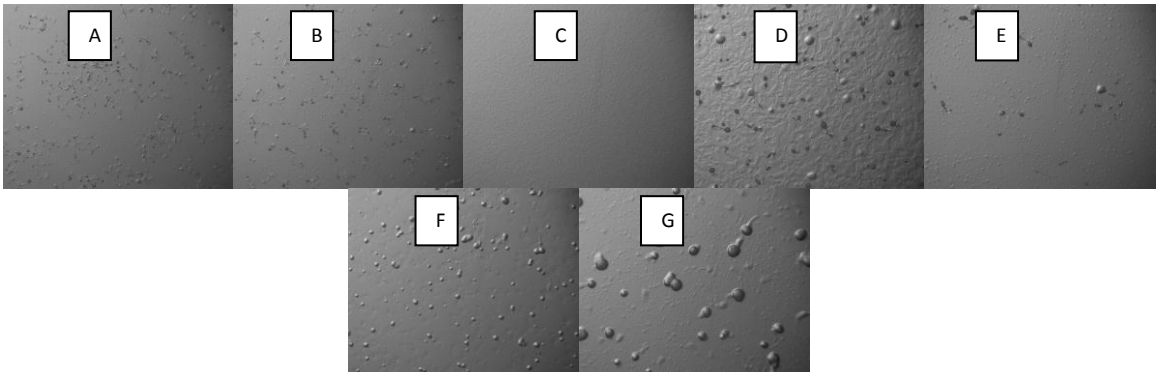


Figure 10: DB Agar Plates: They are listed as follows: A: E4-cAR1.3-GFP, B: WT-cAR1.3-GFP, C: cAR1.3, D: WT-cAR1.3-GFP in extrachromosomal vector pdm304, E: WT-cAR1-GFP-AX2 in extrachromosomal vector pdm304, F: WT-cAR1-GFP-AX2, G: A4-cAR1-GFP-AX2.

Discussion

The phosphorylation of the receptors has been shown to elicit downstream responses, and S299, S301, S303 and S304 have been shown to be important in eliciting these responses. To further characterize this phosphorylation, these serine residues were mutated to alanines and glutamic acid. Alanine was used because of its lack of ability to react with phosphate. Glutamic acid was used because it has oxygen in the phosphate position, and thus cannot be phosphorylated upon cAMP binding to the receptor. Figure 6 shows the expression of all mutants in the cAR1.3 backgrounds. Also, DB agar plates were made at different cell concentrations to test for developmental defects in the mutants. These plates showed the rescue of all cell lines, thus demonstrating the ability of the mutant receptors to function. The cAR1.3 (cAR1/3 knockout) was fully rescued by both E4 and WT. The AX2 cells with both WT and A4 showed an overexpression profile seen in cAR1 wildtype receptor overexpression (Kim 1997).

Cells use chemotaxis to migrate up chemical gradients. As the cells move towards the chemoattractant source, receptors are phosphorylated and may be dynamically exchanged. Our data shows that there is a redistribution of the receptors to the rear of the cell. It is possible that a phosphatase is at work in the rear of the cell and unphosphoryated receptors migrate to the leading edge. This mechanism may take advantage of the highly polarized nature of a cell. Polarity involves an asymmetric alignment of important proteins in the cell, such as PTEN and PI3K, found in the lagging and leading edge, respectively which alter the concentration of various phosphoinositides at the front and rear as well. It is possible that unphophorylated and phosphorylated receptors associate within specific phosphoinositide domains. The phosphorylation state may be critical as WT cells have both sensitive (unphosphorylated) and desensitized (phosphorylated) receptors and can respond directionally to a wide range of cAMP. I speculate that these sensitive and desensitized receptors are turned over and recycled dynamically, as the cell continues to move up a gradient.

The mutants have differences in both sensitivity and localization. The cAR1-A4-GFP cells are unable to phosphorylate at the four mutated residues and are therefore more

sensitive to the gradient. All of the receptors appear to localize to the front of the cell, in the newly formed pseudopodia, as they try to sense the gradient and chemotax towards the needle. The cAR1-E4-GFP cells have a higher level of chemotactic response than cAR1-A4-GFP. They are desensitized and the receptors localize as an internal gradient towards the lagging edge of the cell. The receptors in the cAR1-WT-GFP cells are uniformly distributed on the plasma membrane.

In terms of downstream signaling, the PKB substrate phosphorylation is a great output to use to determine activation of the signaling pathways. The cAR1-WT-GFP cells are expected to be able to activate downstream responses at both high and low concentrations of cAMP, and thus would saturate only at very high concentrations. The cAR1-E4-GFP cells saturate at 100 uM, which is very high, as they need higher levels of cAMP to promote downstream signaling. The A4 cells saturate at 10 uM, a 10-fold decrease in concentration, because they are overly sensitive to the concentration and need less cAMP to propagate downstream signaling.

CHAPTER 3

CONCLUSIONS

The mutations made in the C-terminus of cAR1 have led to a difference in sensitivity and in localization during chemotaxis to cAMP. This was tested in various ways including the examination of signaling pathways in a gradient of chemoattractant, the differential localization, and the saturation of PKB substrate phosphorylation in cells expressing wildtype and mutant receptors.

A future step in this project is to see if the mutants correlate with internalization of the receptor and also electromobility shift to look at the phosphorylation state of the receptor. The main question I set out to answer was how phosphorylation affected sensitivity and adaptation of the receptor to differing concentrations of chemoattractant, but then we made this remarkable discovery that the receptors also seem to redistribute when in a cAMP gradient. When taking into account sensitivity, a loss in ligand binding would also be expected. The A4 cells need less ligand to get response, but they can't detect the gradient, so even in a saturating concentration, they will not phosphorylate, and remain sensitive. The E4 cells are desensitized, which means they need more ligand to elicit a response. These receptors redistribute strongly to the rear, which suggests there is a mechanism that sends phosphorylated receptors rearward. They use an asymmetric distribution and redistribution of receptors to attempt to gain sensitivity across the whole cell. Wildtype receptors also partially relocalize to the rear during chemotax since there are a higher fraction of receptors that are phosphorylated. They respond at all concentrations of cAMP tested.

A critical aspect of this relocalization is that this differential response, if it also occurs in wildtype receptors, would help sharpen the internal responses and help explain the highly localized responses seen in migrating cells. Future directions would include genetically

tagging cm1234, where all serine residues are mutated to alanine and glycine (cAR1/2/3) or deleted (cAR4), and looking for differences when all basal phosphorylation is prevented. This would also highlight the importance of the S299, S301, S302, and S304 residues used in this work.

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