CHEMOREPLLANTS AND THEIR SIGNAL TRANSDUCTION PATHWAYS: Gβ and Akt PROTEINS NECESSARY FOR *Dictyostelium* AprA CHEMOREPULSION

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ALEXANDER PHILIP CONSTANTINE

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ABSTRACT

Chemorepellants and Their Signal Transduction Pathways: Gβ and Akt Proteins Necessary for *Dictyostelium* AprA Chemorepulsion. (April 2013)

Alexander Philip Constantine
Department of Biology
Texas A&M University

Research Advisor: Dr. Richard Gomer
Department of Biology

Cellular movement in response to a chemical gradient, known as chemotaxis, is a critical behavior in many developmental and pathological processes such as embryo development and cancer metastasis. This cell movement can be directed toward a chemical source (chemoattraction), or away from a chemical source (chemorepulsion). In certain normal and tumor mammalian cells, there is secretion of chemorepellant signals that causes specific cell types to move away from them. For example, Semaphorin 3A acts as a chemorepellant involved in axon guidance during neuronal development and is implicated in glial cancer cell metastasis (glioblastoma multiforme), but its mechanism of action is not yet well understood. Studies using the eukaryotic unicellular amoeba *Dictyostelium discoideum* as a model showed that proliferating *Dictyostelium* cells secrete a protein called AprA, and that AprA is an extracellular signal that functions both as a chemorepellant and an inhibitor of cell proliferation. By analyzing the movement of mutant strains in a gradient of recombinant AprA, the role of specific proteins in the AprA chemorepulsion mechanism was assessed. The proteins CrlA, Gα9 and Gβ have been implicated in AprA cell signaling and growth pathways, but not directly in AprA

chemorepulsion signaling. It was found that Gβ, but not Gα9 or CrlA, is necessary for chemorepulsion by AprA. The proteins IplA, Akt1/2, PiaA, Gca/sgcA and PI3K 1/2/3/4/5, are components of other chemotaxis signaling pathways, but are not known to be involved in AprA signaling pathways. This study's results indicate that of these, only Akt1/2 is necessary for chemorepulsion by AprA. These results indicate that Gβ and Akt1/2 proteins are involved in the chemorepellant signaling pathway for AprA in *Dictyostelium discoideum*. The presence of functional homologs of Gβ and Akt1/2 proteins in other cells, for example mammalian neutrophils, suggests that they may be involved in a similar chemorepulsion pathway. Through identifying the components of the transduction pathway of chemorepellants such as AprA, the development of novel therapeutics that can modulate this pathway could help lead to improved treatments in cancer and autoimmune disease.

DEDICATION

To those this research might help in the future.

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CHAPTER I

INTRODUCTION

In physiological events such as embryo development and pathological events such as cancer metastasis, directed cell movement in response to a chemical gradient, known as chemotaxis, is a fundamental behavior [1, 4]. This cell movement can be directed toward a chemical source (chemoattraction), or away from a chemical source (chemorepulsion). Although much is understood about the identity of chemoattractants and the molecular components involved in their signaling, little is known on the identity and signal transduction of chemorepellants.

In certain normal and tumor mammalian cells, there is secretion of chemorepellant signals that causes specific cell types to move away from them [1-3]. For example, Semaphorin 3A acts as a chemorepellant involved in axon guidance during neuronal development and is implicated in invasive glial cancer cell metastasis (glioblastoma multiforme), but its mechanism of action is not yet well understood [2]. Studies using the eukaryotic unicellular amoeba *Dictyostelium discoideum* as a model showed that proliferating *Dictyostelium* cells secrete a protein called AprA, and that AprA is an extracellular signal that functions both as a chemorepellant as well as an inhibitor of cell proliferation [5-7]. Using this model organism, a better understanding of chemorepulsion signaling could be possible.

Previous research using *Dictyostelium* has also informed our understanding of the chemoattractant mechanism of directed cell movement. In *Dictyostelium* cells, when the concentration of a chemoattractant is higher at the leading edge of the cell versus its trailing

edge, rearrangement of cytoskeletal components takes place allowing pseudopod formation in the direction of the chemoattractant [8]. Correspondingly in mammalian neutrophils, similar pathways that lead to cytoskeletal rearrangement and movement towards the chemoattractant were observed [8-11]. Experiments using the *Dictyostelium* chemoattractant cAMP showed that this response was mediated by a ligand-induced G-protein-coupled receptor that activates and induces translocation of phosphatidylinositol 3-kinase (PI3K) to the leading edge of the cell [8-11]. PI3K activation continues a series of reactions that polarize pseudopod formation in the direction of the chemoattractant [12, 13]. In the front of the cell phosphorylation of phosphatidylinositol $(4,5)P_2$ (PIP2) lipids to phosphatidylinositol $(3,4,5)P_3$ (PIP3) occurs, leading to actin polymerization and pseudopod formation at the leading edge [8, 12-16]. Reciprocally, in the back of the cell the phosphatase PTEN dephosphorylates PIP3 to PIP2 [8, 12-14]. This reciprocal localization of PIP3 in the front of the cell and PIP2 in the back of the cell, to reduce pseudopod formation, has been observed in both *Dictyostelium* cells and neutrophils [15, 17, 18]. Understanding the signal transduction pathway of chemoattraction might give insight into the pathway used by chemorepellant signals.

Previous studies indicate that when *Dictyostelium* cells are at high density, the secreted chemorepellant AprA facilitates colony spreading [7]. It has been shown in the literature that a recombinant AprA (rAprA) source is sufficient for repulsion activity [7]. Previous findings indicate that AprA signals through a G-protein coupled transmembrane receptor and that the proteins Gα8 and QkgA are necessary for the transduction of the chemorepellant and cell-proliferation inhibition AprA signal [7]. Proteins such as BzpN that are necessary for the cell-proliferation inhibition activity of AprA, are not involved in the chemorepellant activity of AprA

[7]. A key aspect of this research is to test whether signal transduction components that mediate the proliferation-inhibiting activity of AprA or signal transduction components known to function in chemoattractant signaling are also involved in chemorepellant signaling. Previously, 12 proteins have been identified that are implicated in the chemoattractant pathway, in the ability of AprA to inhibit proliferation, or are involved in other chemotaxis pathways (Appendix A, Table 1). Four of these proteins (Gα8, CfaD, QkgA, and PakD) have been found to be necessary for chemorepulsion from recombinant AprA. To test the hypothesis that the other 8 proteins are also necessary for chemorepulsion we will examine whether mutant cells lacking specific proteins are still repelled by rAprA. The proteins Gα9, Gβ and CrlA are components involved in AprA signaling pathways, most notably involved in the ability of AprA to inhibit cell proliferation. Proteins IplA, Akt1/2, PiaA, Gca/sgcA and PI3K 1/2/3/4/5, are components of chemotaxis signaling, but were not known to be involved in the AprA signaling pathway. This study will help to identify the components of the signal transduction pathway that mediate chemorepulsion. From this identification of the components of this pathway, new potential targets for therapeutics could be found. In disease states involving cell repulsion such as cancer cell metastases, being able to inhibit cell repulsion could help manage a patient's health.

CHAPTER II

METHODS

Cell culture and recombinant AprA

Multiple mutant strains of *D. discoideum* were used in determining the components of the signaling pathways for the chemorepellant AprA. The strains AX2 wild-type, c*rlA*$^{-}$ [DBS0235627] [19], *akt1*$^{-}$*/2*$^{-}$ [DBS0236785] [20], *gα9*$^{-}$ [DBS0236109] [21], *gβ*$^{-}$ [DBS0236531] [21], *iplA*$^{-}$ [DBS0236260] [22], *piaA*$^{-}$ [DBS0304637] [23], *gca*$^{-}$*/sgcA*$^{-}$ [DBS0302679] [24] and *pi3k* 1^{-}/2^{-}/3^{-}/4^{-}/5^{-} [DBS0252652] [25] were grown in Formedium HL5 axenic shaking culture media following the description in [26]. Recombinant AprA protein was made using *Escherichia coli* as described in [27].

Under-agarose cell movement

In measuring the effect of AprA on directing *Dictyostelium* chemotaxis under-agarose cell movement assays were performed according to the methods described in SI Methods and Materials of [7]. Cells from culture were diluted to 1×10^4 cells/mL in HL5. The agarose plates were made with a solution of 1% low-electroendosmosis molecular biology-grade agarose (Fisher, Pittsburgh, PA) in SM media (in 1 L ddH_2O: 10 g glucose, 10 g proteose peptone, 1 g yeast extract, 1 g $MgSO_4$*7H_2O, 1.9 g KH_2PO_4, 0.6 g K_2HPO_4, pH 6.2) microwaved until boiling. 20-mL volumes of this solution were transferred into 100 × 20 mm petri plates and allowed to cool/solidify. Using a razor blade, a 2- × 44-mm rectangular hole was cut into the center of the agarose. 200-μL of diluted cell culture of the indicated genotype was added in the hole. The plates were incubated overnight at 20°C. The culture dish was then placed on an

inverted microscope, and 200 μL of HL5 media was added to the hole. A 20× objective was used to image cells beneath the agarose ~1.5 mm away from the hole. Multiple frames across the agar plate were captured.

Insall chamber assay

To measure cell movement in response to a rAprA gradient, an Insall chamber assay was performed. For each strain, 300 μL of cells, diluted in HL5 media to a concentration of 2 x 10^5 cells/mL, were pipetted onto a coverslip and allowed to adhere for 1 hour in a humid box at 20°C. An Insall chamber slide was used for visualization of cell movement (Appendix A, Figure 1). 200 μL of HL5 media was placed into both wells of the Insall slide. After allowing cells to adhere, the media on the coverslip was removed. The coverslip was then placed on the Insall slide, ensuring that the two extending ports were left accessible (Appendix A, Figure 1). Tissue paper was then used to carefully absorb excess media around the cover slip without moving its position and disturbing the cells. The HL5 media from the outer well was removed using a micropipette and replaced with 70 μL of 2,000 ng/mL rAprA protein in HL5 to establish a rAprA concentration gradient in HL5 (rAprA in the outer well, HL5 media in the inner well). Cells adhered to the coverslip could now be visualized moving in response to the gradient on the raised "bridge" separating the inner and outer wells of the chamber slide. A time-lapse video for 2 hours was made using an inverted microscope with a 10x objective lens to assess cell movement in response to the created gradient as described in [7]. The video recording was then used to manually track cell movement. Cell tracking data collection was started 20 minutes into the film to allow time for establishment of the rAprA gradient. Approximately 10 cells per assay were tracked/measured for the distance and angle they moved on the film within a 1 hour time

window. For each cell a line was drawn from the initial point to the final point and measured for distance. The angular direction of the line relative to the rAprA gradient was measured from the initial and final position of the cell tracked. Data was entered into Excel and distance measurements were separated into the x and y axis (Cos(angle)*distance, Sin(angle)*distance). The x-axis distance value was converted into microns (to assess directional chemotaxis in the x-plane: away or towards rAprA).

Statistics

All statistical analyses were done using Prism (GraphPadSoftware, La Jolla, CA). Unless otherwise noted, one-way ANOVA was used to compare between multiple groups, and Student's *t* test was used to compare between two groups.

CHAPTER III

RESULTS

AprA facilitates cell spreading

Previous studies with *Dictyostelium discoideum* have shown that proliferating *Dictyostelium* cells secrete AprA, and have indicated that when *Dictyostelium* cells are at a high density, the secreted chemorepellant AprA facilitates colony spreading [7]. To show the chemorepellant effect of AprA qualitatively, under-agarose cell movement was assessed in AX2 wild-type *Dictyostelium* cells and *aprA*$^{-}$ mutants. Cells were observed from the edge of the well cut in the agar plate, and across the plate. Wild-type cells, previously shown to secrete AprA, with HL5 media placed in the well showed significant colony spreading across the plate (Figure 1). *aprA*$^{-}$ mutants with HL5 media placed in the well showed significantly diminished movement away from the edge of the well (Figure 1). An explanation for this observed movement is that AprA functions as a chemorepellant, causing cell spreading when *Dictyostelium* cells are in a high density or colony of cells. In the absence of AprA production, this chemorepulsive effect is significantly diminished (Figure 1).

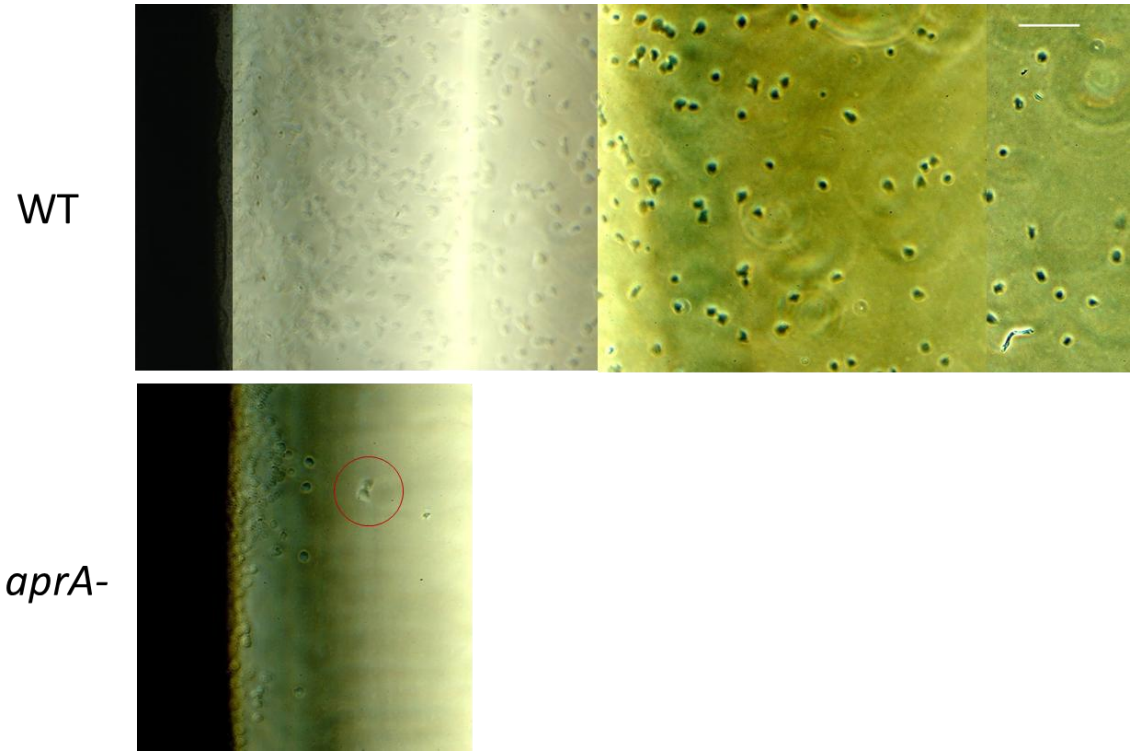


Figure 1. AprA facilitates cell spreading. Composite under-agarose cell displacement images. Diluted cultures of cells of the indicated genotype were placed in a 2 × 44-mm rectangular well cut into a layer of agarose in a cell culture dish and incubated over a 24h period. A 20× objective was used to image cells beneath the agarose ~1.5 mm away from the well (well can be seen as darkened far-left portion of the image). Multiple consecutive frames across the agar plate were captured and used to create single images displayed to show the cell distribution. Encircled *aprA*$^{-}$ cells highlight the furthest cells displaced in the *aprA*$^{-}$ sample. Scale is bar 10μm.

Gβ, but not Gα9 or CrlA, is necessary for chemorepulsion by AprA

The protein AprA inhibits cell proliferation and acts as a chemorepellant [7, 27]. The 8 proteins selected for analysis have been previously implicated in cellular proliferation via AprA signaling

or other chemotaxis signaling pathways (Appendix A, Table 1). To assess their involvement in the AprA chemorepulsion pathway, mutant cell lines lacking the specific protein studied were utilized. By analyzing the movement of several mutant strains in an Insall chamber assay, a determination of the necessity of these proteins in AprA chemorepulsion signaling was possible.

The proteins CrlA, Gα9 and Gβ have been implicated in AprA cell signaling pathways and chemoattractant signaling, but not directly in the AprA chemorepulsion pathway [28-30] . To assess these proteins' necessity in the AprA chemorepulsion signal transduction pathway, mutant cell lines of these proteins were examined for repulsion from a source of rAprA via an Insall chamber assay. From previous studies it was established that wild-type cells subjected to rAprA showed a significant movement away from the rAprA source [7]. Wild-type cells, in the HL5 buffer control condition, showed no significant bias in movement (Figure 2). A bias in movement away from the source of rAprA was observed for c*rlA*$^{-}$ and *gα9*$^{-}$ mutant cells, comparable to WT + rAprA conditions (Figure 2). However, *gβ*$^{-}$ cells showed no significant movement away from the source of rAprA, compared to wild-type (Figure 2). Unpaired t-test analysis showed that in comparison with the WT + HL5 control, *gα9*$^{-}$ cells showed a statistically significant displacement away from the rAprA source. Results in a t-test comparing WT control and c*rlA*$^{-}$ displacement showed that c*rlA*$^{-}$ mutant cells did not have a statically significant displacement away from a rAprA source. However, paired t-test results with a value of 0 (0 average cell displacement), did yield results for significant displacement away from the rAprA source in c*rlA*$^{-}$ mutant cells. Unpaired t-test in comparison with the WT +HL5 control showed that *gβ*$^{-}$ cells did not have a significant displacement bias away from the rAprA source. These

results point toward Gβ being necessary for AprA chemorepulsion signaling, whereas CrlA and Gα9 are not necessary.

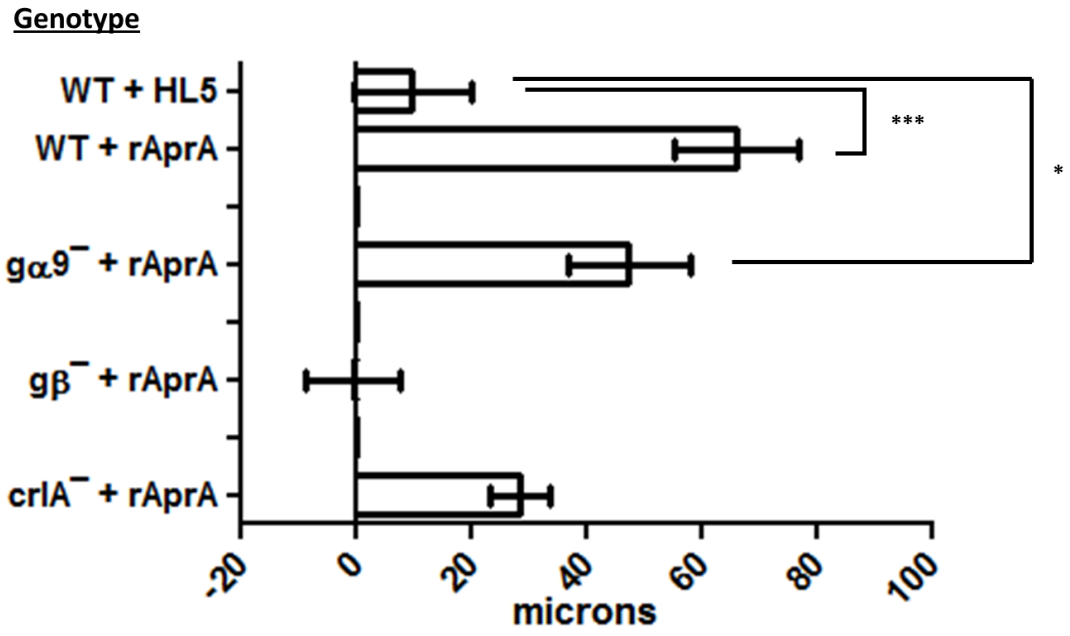
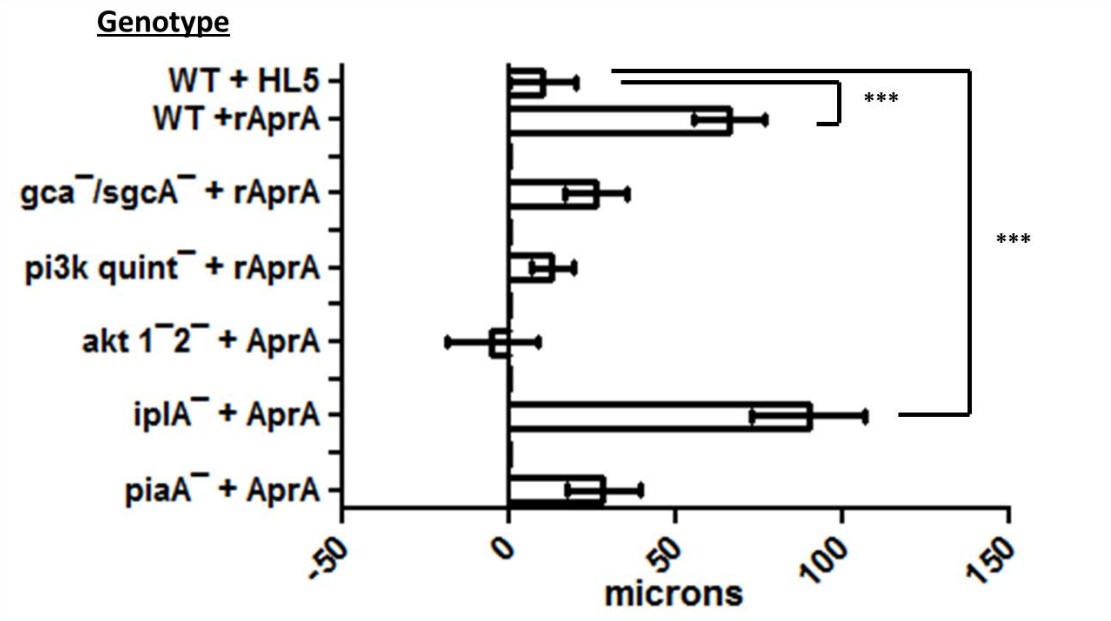


Figure 2. Gβ, but not Gα9 or CrlA, is necessary for chemorepulsion by AprA. Cells of the indicated genotype in HL5 media were placed near a rAprA source or HL5 buffer using an Insall chamber. Cells were able to move in the rAprA gradient, and their displacement was filmed over a 1h time period. Directional data away from the rAprA source was measured for individual cells. The average cell displacement in microns, from the source of rAprA, is shown. Average cell displacement was found through 3 independent experiments, measuring 10 cells/experiment. Positive values indicate movement away from the rAprA source. For *crlA*$^{-}$ + rAprA, displacement was measured against a value of 0 in a paired t-test. A statistically significant difference in average displacement against the value of 0 was found ($P<0.0001$). *$P<0.05$ ***$P<0.001$ t-test.

Akt is necessary for chemorepulsion by AprA

The proteins IplA, Akt1/2, PiaA, Gca/sgcA and PI3K 1/2/3/4/5 analyzed are components of chemotaxis signaling, but are not known to be involved in an AprA signaling pathway (Appendix A, Table 1). *akt1*$^{-}$*/2*$^{-}$ and *pi3k 1*$^{-}$*/2*$^{-}$*/3*$^{-}$*/4*$^{-}$*/5*$^{-}$ mutants had minimal movement bias in response to the rAprA gradient, compared with the WT + HL5 control (Figure 3A). In comparison with the wild-type + HL5 control, *pi3k 1*$^{-}$*/2*$^{-}$*/3*$^{-}$*/4*$^{-}$*/5*$^{-}$ mutants seemed to show little bias in response to rAprA (Figure 3A). However, the slow movement phenotype of this mutant affected the overall displacement results when *pi3k 1*$^{-}$*/2*$^{-}$*/3*$^{-}$*/4*$^{-}$*/5*$^{-}$ mutants were subjected to the rAprA gradient. Comparing the *pi3k 1*$^{-}$*/2*$^{-}$*/3*$^{-}$*/4*$^{-}$*/5*$^{-}$ mutant + HL5 control condition (instead of the WT + HL5 control) to the *pi3k 1*$^{-}$*/2*$^{-}$*/3*$^{-}$*/4*$^{-}$*/5*$^{-}$ mutant + rAprA results, statistically significant movement away (chemorepulsion) from the rAprA source was observed (Figure 3B). Mutant lines g*ca*$^{-}$*/sgcA*$^{-}$, *iplA*$^{-}$ and *piaA*$^{-}$ showed movement away from the rAprA source (Figure 3A). However, t-test analysis showed that only *iplA*$^{-}$ cells had statistically significant displacement away from the rAprA source, when compared with the WT + HL5 control. In a paired t-test with a value of 0 (0 average cell displacement), g*ca*$^{-}$*/sgcA*$^{-}$ and *piaA*$^{-}$ mutant cells showed significant displacement away from the rAprA source. Therefore, *akt1*$^{-}$*/2*$^{-}$cells were the only mutants that showed no statistically significant displacement away from the rAprA source, implicating the Akt protein's necessity in AprA chemorepulsion signaling, whereas the other proteins are not necessary.

(A)


Genotype
WT + HL5
WT +rAprA
gca⁻/sgcA⁻ + rAprA
pi3k quint⁻ + rAprA
akt 1⁻2⁻ + AprA
iplA⁻ + AprA
piaA⁻ + AprA
***
***
-50
0
50
100
150
microns

(B)

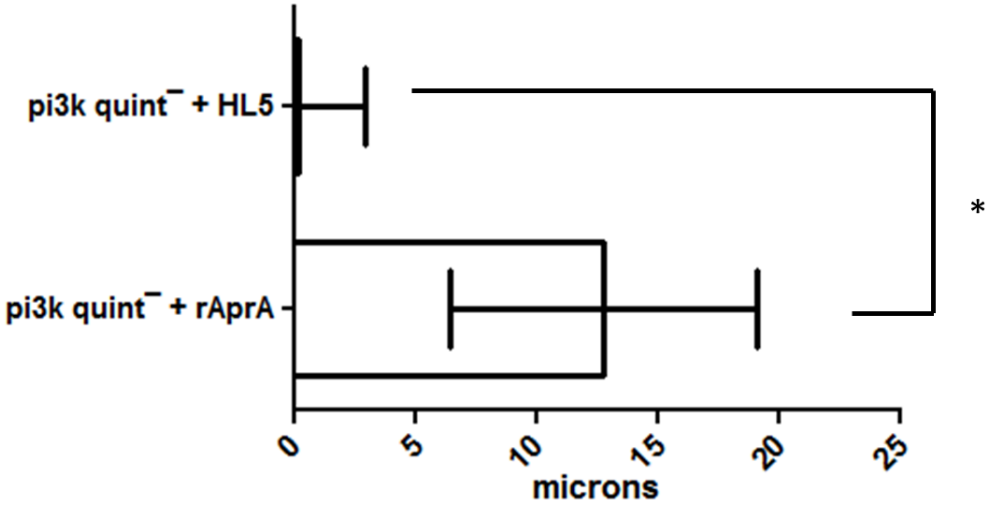

pi3k quint⁻ + HL5
pi3k quint⁻ + rAprA
*
0
5
10
15
20
25
microns

Figure 3. Akt is necessary for chemorepulsion by AprA. (A) Cells of the indicated genotype in HL5 media were placed near a rAprA source or HL5 buffer using an Insall chamber. Cells were able to move in the rAprA gradient, and their displacement was filmed over a 1h time period. Directional data away from the rAprA source was measured for individual cells. The average cell displacement in microns, from the source of rAprA, is shown. Average cell displacement was found through 3 independent experiments, measuring 10 cells/experiment. Positive values indicate movement away from rAprA source. For *gca*$^{-}$ */sgcA*$^{-}$ and *piaA*$^{-}$ + rAprA samples, displacement was measured against a value of 0 in a paired t-test. A statistically significant difference in average displacement against the value of 0 was found for both ($P<0.05$). ***$P<0.001$ t-test. **(B)** *pi3k quint*$^{-}$ data, separated for clarity. t-test comparison of *pi3k quint* $^{-}$ + rAprA and *pi3k quint* $^{-}$ + HL5 media, shows significant displacement. * $P<0.05$ t-test.

CHAPTER IV

CONCLUSIONS

The results presented support the function of AprA as a chemorepellant, and its function in facilitating cell spreading [7]. Additionally, the findings presented show that the G-protein, Gβ and the kinase Akt 1/2 proteins are necessary in the AprA chemorepulsion signaling pathway. Furthermore, these results show that the proteins Gα9, CrlA, IplA, PiaA, Gca/sgcA and PI3K 1/2/3/4/5 are not necessary for AprA's chemorepellant function.

These results implicating the necessity of Gβ in AprA chemorepulsion activity of *Dictyostelium* cells also link the AprA chemorepulsion signaling pathway to AprA's function as a cell proliferation inhibitor [30]. $g\beta^{-}$ cells did not remain sensitive to rAprA signaling, indicating that the presence of Gβ in the cell is necessary for AprA chemorepulsion activity. Additionally, it has been shown that Gβ is necessary for AprA-stimulated GTP binding, linking AprA chemorepulsion signaling to G-protein signaling and potentially a cAMP-like chemotaxis pathway [30]. The Gβ subunit of eukaryotic G-protein coupled receptors is important in transducing extracellular signals [31]. Human cells have many Gβ subunits [32]. These newly presented results implicate Gβ signaling involvement in mammalian cell chemorepulsion. It may also be likely that AprA signals via a transmembrane receptor linked to a G-protein, activating GTP binding to a Gα subunit (requiring Gβ) and activating effector proteins like Akts to transduce the chemorepulsive signal in *Dictyostelium*.

The Akt protein, also known as protein kinase B (PKB), has two forms in *Dictyostelium* (Akt 1/2) that serve redundant functions. Akt/PKB proteins in *Dictyostelium* are known to function in chemotaxis, cell polarity, and in cAMP chemoattraction via G-protein coupled receptors and PI3-kinases [20]. From the results presented it was shown that PI3K 1/2/3/4/5 proteins were not required for AprA chemorepellant activity. This suggests that Akt is activating other downstream proteins, not PI3-kinases, to signal chemorepulsion. The specific PKBR-1 protein has been linked to morphogenesis during *Dictyostelium* multicellular development [20]. Along with the results found in this study, chemorepellants like AprA may function in a pathway that also overlaps in cell survival and morphogenesis signaling. Mammalian cells have a homologous Akt/PKB protein that is also known to function in chemoattraction in addition to cell survival [33]. In human cells the connection between G-protein coupled receptors and Akt protein activation has been made [33]. In context of the results found in this study of AprA chemorepulsion signal transduction, it may be possible that mammalian chemorepellants also bind transmembrane receptors coupled with G-proteins, activating subsequent Gα-GTP binding, activating an Akt protein pathway that functions in chemorepellant signaling and cell survival.

AprA's function as a chemorepellant may have been selected to allow the spreading of cells across a larger area, obtaining nutrients and thereby allowing greater growth and survival. Similarly, the function of chemorepellants in mammalian axon guidance during neural development may have been selected and exploited by neural cancer cells in order to facilitate cell organization and survival. Using *Dictyostelium* as a model organism allows for a highly tractable study of these conserved mechanisms of chemotaxis seen across many different organisms and cells. In studying the AprA signaling cascade, translation to similar mammalian

signaling pathways is possible. Therefore, by elucidating the steps in the AprA chemorepulsion signaling pathway, insight into new molecular targets to inhibit cancer metastasis and development of new methods for treating chemorepellant-related pathologies could be possible.

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APPENDIX A

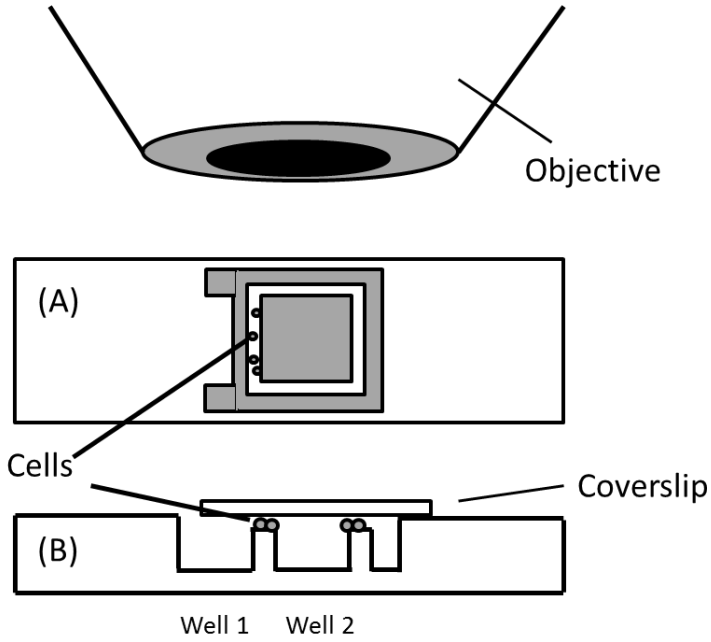

Figure 1. Insall chamber slide views. (A) Top view of Insall chamber slide showing depressed regions in gray and raised regions in white. Depressed regions served as wells for media/rAprA conditions to establish chemorepulsion gradient. Raised regions served as platforms between conditions in which cells could move in response to the rAprA gradient. **(B)** Side view of Insall chamber slide, showing cells and coverslip and highlighting depressed wells and raised "bridges" for chemorepulsion analysis. Region not covered by coverslip indicates ports used to extract/add media.

Table 1. Proteins known to be involved in AprA or other chemotaxis signaling pathways.

***Dictyostelium* protein**	**Similar to human Protein**	**Human protein function**	**References**
CfaD [34]	Cathepsin L	Secreted cysteine protease	[35]
CrlA [28]	7-transmembrane G protein-coupled receptors	Signal transduction	[29]
Gα8 [30]	Gα proteins	Signal transduction	[32]
Gα9 [30]	Gα proteins	Signal transduction	[32]
Gβ [30]	Gβ proteins	Signal transduction	[32]
QkgA [36]	ROCO kinases LRRK1, LRRK2	Down regulated in some leukemias; affects neurite extension in rats	[37-40]
Akt 1/ 2 [20]	Akt/Protein Kinase B – serine/threonine kinase	Chemoattraction, signal transduction, metabolism, angiogenesis	[33, 41, 42]
PakD	PAK family of serine/threonine kinases	Motility, proliferation; overexpressed in some tumors	[43]
PiaA [44]	Tor Complex 2- Rictor subunit	Motility, growth, actin cytoskeleton modulation	[45]
IplA [46]	IP_3 Receptor - Inositol 1,4,5-trisphosphate receptor protein	Ca^{2+} -release channel, cytoplasmic Ca^{2+} signaling	[47]

Dictyostelium protein	Similar to human Protein	Human protein function	References
Gca/sgcA [24, 48]	Adenylyl cyclase	Signal transduction	[49]
PI3K quint [25]	Phosphatidylinositol 3-kinases	Chemoattraction, migration	[8-11]