RICE UNIVERSITY

AprA: An Autocrine, Secreted Factor that Represses Cell Proliferation in *Dictyostelium discoideum*

by

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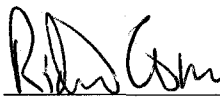
A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Master of Arts

APPROVED, THESIS COMMITTEE:



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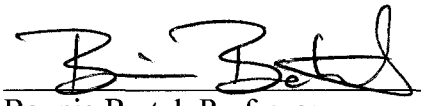

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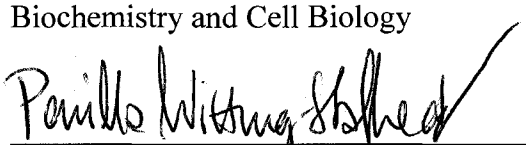

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ABSTRACT

AprA: An Autocrine, Secreted Factor that Represses

Cell Proliferation in *Dictyostelium discoideum*

by

Jonathan Myongwoo Choe

Dictyostelium cells secrete factors during growth and starvation. AprA (autocrine proliferation repressor), a 60 kDa protein secreted as part of a 150 kDa protein complex, functions as a cell proliferation repressor. In the present study, we expressed recombinant AprA (rAprA) in bacteria. We found that rAprA is bioactive as a proliferation repressor and that rAprA binds to live *Dictyostelium* cells. rAprA, when added to the growth medium of wild-type and *aprA*$^-$ cells, slows the proliferation of these cells; however, rAprA does not slow the proliferation of *crlA*$^-$ or *cfaD*$^-$ cells. CrlA is a putative receptor, and CfaD is a protein secreted by cells. These findings indicated that rAprA needs CrlA and CfaD to act as a proliferation repressor. However, cells lacking CrlA bound rAprA with high affinity, suggesting that CrlA may not be the receptor for AprA. Thus, AprA binds to cell surface receptors in a signal transduction pathway that involves CrlA and CfaD to negatively regulate cell proliferation.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Richard Gomer, for taking me in his lab and for putting me on this project. Many thanks go to Dr. Deenadayalan Bakthavatsalam and Dr. Yitai Tang for their endless hours of training me in *Dictyostelium* and general lab techniques, for their patience, and for enhancing my graduate education. I would also like to thank my labmates: Dr. Darrell Pilling, Dr. Kevin Houston, Jeffrey Crawford, Neda Nikravan, Anu Maharjan, and Varsha Vakil for their friendship, for their hard work, and for making the lab an enjoyable place to work. To my lab trainees: Curtis Lin, Tara Skebba, Valerie Peicher, and Wendy Zhang, I appreciate your efforts, eagerness to learn, and loyal dedication. To Peter Nguyen, Peng Zhai, Shan Gao, Shirley Liu, and Mary Harrison, thank you for making my transition to Rice a pleasant one. To my classmate and close friend, Todd Mollan, thank you for your friendship and for all of the advice that you have given me. Thank you to my committee members: Dr. Bonnie Bartel, Dr. Michael Stern, Dr. Pernilla Wittung-Stafshede, and Dr. Rob Raphael for challenging me to become a better scientist and for their guidance throughout my project. Last but definitely not least, I would like to thank my wife Jennifer Hsing Choe and my daughter Eva Isabella Choe for their undying love and support. I am truly blessed.

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Introduction

Although many discoveries have been made regarding the factors and mechanisms involved in cell group size regulation, much remains to be understood regarding how cells regulate their own proliferation and thus, limit tissue size. A number of studies have examined the hypothesis that cells secrete factors that regulate tissue size. For example, counting factor (CF), a secreted 450 kDa protein complex, regulates cell group size in *Dictyostelium* by repressing cell to cell adhesion (Roisin-Bouffay et al., 2000) and increasing cell motility (Tang et al., 2002). Thus, as *Dictyostelium* cells aggregate, factors are in place to ensure that optimal group size is maintained. Study of the components present in partially purified CF revealed a novel 60 kDa protein that was not a CF component (Brock and Gomer, 2005). This novel protein was named AprA for autocrine proliferation repressor.

AprA is a signal secreted by *Dictyostelium* cells that slows the proliferation of cells and is a component of a 150 kDa complex (Brock and Gomer, 2005). *Dictyostelium* cells that do not secrete AprA proliferate at a faster rate than wild-type cells, while cells that secrete too much AprA tend to proliferate at a much slower rate than wild-type cells (Brock and Gomer, 2005). To study the proliferation inhibition property of AprA, AprA was immunoprecipitated and added to the growth media of wild-type and *aprA*$^-$ cells. Exogenous immunoprecipitated AprA added to wild-type cells at a concentration of 10 ng/ml inhibited cell proliferation by 33% (as compared to HL-5 medium control), while exogenous AprA inhibited cell proliferation by more than 50% in *aprA*$^-$ cells (Brock and Gomer, 2005). The focus of my research is to characterize the function of AprA by expressing a recombinant version of the protein and to study the AprA signal

transduction pathway. By studying the signal transduction pathway of AprA, we may be able to use the knowledge gained to understand signal transduction pathways involved in cell proliferation regulation in other organisms.

Tissue size regulation via secreted factors

How do tissues know when to stop growing? The answer to this question has been investigated for many years. Current knowledge suggests that the concentration of diffusible factors allows cells to sense the size of a specific group of cells or to sense the number of cells within a specific environment (Gomer, 2001). To effectively sense the size of a specific group of cells, the secreted factor diffuses away from the cells. The factor concentration increases as the number of cells in the group increases (Yuen and Gomer, 1994). If the factor inhibits proliferation at a concentration X, the cells will stop proliferating when there are enough cells to generate the concentration X of the factor. For example, the mammalian liver has the ability to regenerate to its original size if any portion of the liver is damaged or removed (Micalopoulos and DeFrances, 1997). The spleen is another example of a tissue that is negatively regulated by secreted factors. In a 1964 experiment, the spleen was removed from a mouse. Fragments of this spleen were then transplanted into different sites of a splenectomized, syngeneic mouse. The end result was that the spleen fragments grew to the correct weight of a normal mouse spleen (Metcalf, 1964). This study suggested that some factor existed that mediated communication between the different spleen fragments to grow to the right size and to tell the cells when to stop proliferating. The search for the identities of these factors is still on-going.

Autocrine regulation

There are a few examples of autocrine regulation where cell proliferation is negatively regulated. For example, myostatin is a 25 kDa protein that is a member of the TGF beta superfamily of protein that is made by and secreted by myoblasts (Lee and McPherron, 1999). Myostatin concentrations rise when the amount of muscle in a body increases (Lee and McPherron, 1999). High concentrations of myostatin inhibit myoblast proliferation, which keeps the amount of muscle in the body at a constant level (Thomas et al., 2000). This observation was supported in a study where mutated myostatin resulted in abnormally large muscles in animals (McPherron et al., 1997). Thus, muscle size appears to be regulated by a negative feedback loop where a secreted factor, myostatin, is used to sense the number of secreting cells and to inhibit the proliferation of the secreting cells (the myoblasts). Another example of a pathway regulated by a negative feedback loop is thyroid size regulation. Thyroid cells normally secrete thyroid hormone, which inhibits the release of thyroid-stimulating hormone (TSH) from the pituitary. TSH functions to stimulate the growth of the thyroid. Thus, if the thyroid is damaged, thyroid hormone levels fall, allowing more TSH release to promote thyroid growth (DuMont et al., 1989). Another example of autocrine regulation via a negative feedback loop involves regulation of adipose tissue within the human body. Leptin, a 16 kDa protein hormone, is secreted by adipocytes and signals the amount of adipose tissue present in the body (Schwartz et al., 2000). When leptin levels rise, leptin interacts with components of the hypothalamus to signal to the body that appetite is satisfied. Once appetite is decreased, adipose tissue accumulation falls, completing the feedback loop. While there are many pieces of evidence supporting the existence of factors that

negatively regulate cell proliferation of many different tissues, only a few of these specific factors have been identified. Identifying these factors will aid in our understanding of tissue size regulation.

***Dictyostelium discoideum* as a model system to study cell group size regulation**

The overall simplicity of *Dictyostelium discoideum* and its ease of use make the organism a great model system to study the regulation of cell group size and proliferation. *Dictyostelium* is a unicellular eukaryote that feeds on bacteria. The cells are haploid and increase in number by binary fission. Once a *Dictyostelium* cell starves, it stops dividing and secretes an 80 kDa glycoprotein called conditioned medium factor (CMF) (Jain et al., 1992). When CMF concentrations exceed approximately 0.3 ng/mL, pulses of cyclic AMP are released as a chemoattractant to start cell aggregation (Jain et al., 1992). Aggregation occurs once the cells have been starving for 5 to 10 hours. The aggregating cells break up into groups of about 20,000 cells using CF as a group size regulator (Roisin-Bouffay et al., 2000). The purpose of *Dictyostelium* cells breaking up into groups of a specific size is to ensure that spores are properly dispersed. During times of starvation, *Dictyostelium* cells aggregate into fruiting bodies, which consist of a stalk and a spore mass (Gomer and Ammann, 1996). If the fruiting bodies are too big, the spore mass will fall over leading to the failure of *Dictyostelium* spores to disperse and find better living environments where food is abundant. Fruiting bodies that are too small are also unable to disperse properly. The spores in these fruiting bodies are too close to the ground so the spores cannot take advantage of the wind to spread over long distances.

During the study of fruiting body formation, it was found that *smlA*$^-$ cells oversecreted CF, which signals to *Dictyostelium* cells to break up into many small groups (Brock and Gomer, 1999). CF was partially purified and found to elute at 450 kDa. It was also found that CF is composed of at least 4 components: CF45, CF50, CF60, and countin. When the genes encoding any of these components are disrupted, large fruiting bodies are observed (Brock et al., 2002).

AprA: A secreted autocrine factor that represses cell proliferation

With some of the CF components known, work was done to distinguish between true CF components and contaminants. A 60 kDa protein was observed in partially purified counting factor (Figure 2). This novel protein was originally named *ncf*60 for "not counting factor" (Brock and Gomer, 2005). The unknown protein was cut from the gel and sent for sequencing at the Baylor College of Medicine core facility. Sequencing results were compared to the *Dictystelium* genome bank to obtain the predicted amino acid sequence (Figure 1). A partial amino acid sequence of the protein (amino acids 14-34) was then used to generate affinity-purified rabbit polyclonal anti-AprA antibodies. To verify and identity the novel protein, a western blot was performed using anti-countin, anti-CF50, and anti-AprA antibodies on the fractions from sieving gel chromatography of conditioned medium (Figure 2). The anti-AprA antibodies only bound to a 60 kDa protein present in the 150 kDa fraction. By using conditioned starvation medium from wild-type cells for this experiment, it was determined that AprA was a secreted protein that was not a component of CF.

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  1 MSKLLILLLL SLVASIFSTP LDDYVNAPDD TYKWTLNNTI EYETFTGYIL ELTSQTWMAE
 61 KSDWPVWKHW VSICVPKGVT TTTTFIYVDG GSNDNWKVPG SMDQTIEIVC LSSGSVSVGL
121 TQIPNQPIIF NNDGVQRFED DLVAYTWRQF LGNTSEPLWL ARLPMTKAVV KCMDAVQEFG
181 KTIGYNSENF VIAGASKRGW TTWLAGVVDP RIIAIVPIVM PILNMIPNMG HQFYAYGEWS
241 FALNDYTGQG VMDYLNGPQM VELAAIVDPF SYRDRYTMPI YAIASSDDEF FLPDSPQFFW
301 NNLTATPEKH LRIVPNAEHS LMGHQIDIIL SIVTFVRLLI TNQPRPTFTW DITYSEDLNS
361 GTIVLTVPEG GIIPYKVKVW TAVTESTTRR DFRIITCMDI TKCIQFIIWD PSDITPTSTG
421 VYSITLSKPD AGWRAFFLEA EYLYAKNSID DEYTLKFTSE VAIVPNTLPF GSCSEYNACG
481 DGSQGDSASS TATL
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Figure 1. The predicted amino acid sequence of AprA. The gray boxes represent amino acid sequences from tryptic peptides of AprA. The SignalP program indicates that amino acids 1-18 are a signal sequence while the asterisks represent potential N- and O-linked glycosylation sites. The broken underline points to regions that have some similarity to human proteins (Brock and Gomer, 2005).

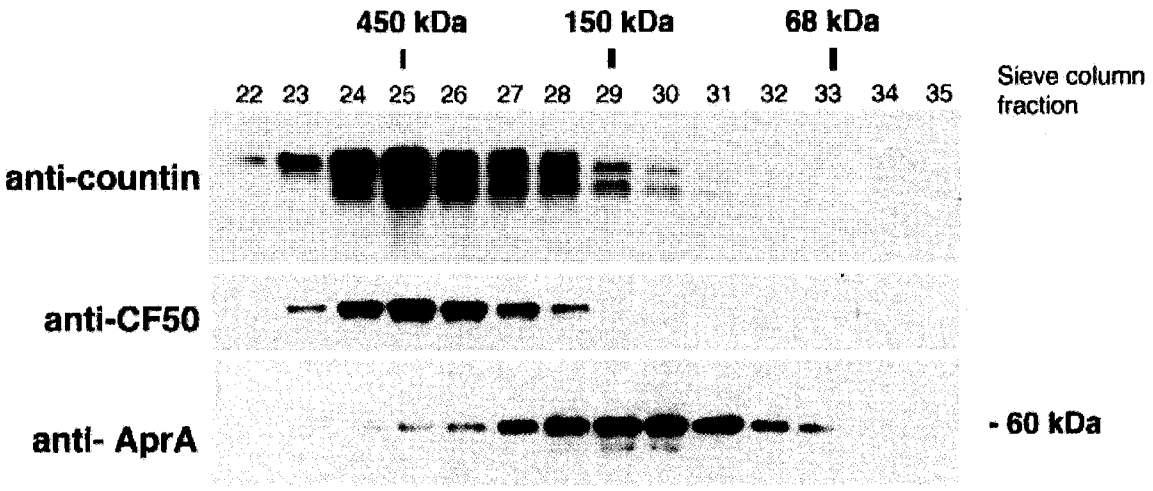

Figure 2. AprA is secreted by starving *Dictyostelium* cells but is not part of the counting factor complex. Conditioned starvation medium from wild-type cells was fractionated by sieving gel filtration chromatography. Western blots were stained with affinity-purified rabbit antibodies against CF50, countin, and AprA. On the SDS-polyacrylamide gels, the upper band stained with anti-countin antibodies is at 40 kDa, the CF50 band is at 50 kDa, and the heavy AprA band is at 60 kDa. The affinity-purified anti-AprA antibodies stained a very minor band at 56 kDa in fractions 28-30. Other than this band and the heavy band at 60 kDa, the anti-AprA antibodies did not stain any other band (Brock and Gomer, 2005).

To study the function of AprA, *aprA*$^-$ cells were made using homologous recombination. A rescue construct was also made in knockout cells by expressing AprA under the control of the *Dictyostelium* actin15 promoter. To understand AprA's role during cell proliferation, knockout, rescued, and wild-type cells were diluted to about 200,000 cells per ml and grown in shaking culture for several days. Cell densities were measured and recorded daily. The data showed that, compared to wild-type cells, *aprA*$^-$ cells proliferated at a faster rate while *aprA*^{OE} cells proliferated much more slowly (Brock and Gomer, 2005). These observations suggested that AprA inhibited cell proliferation. To test this observation, exogenous immunoprecipitated AprA was added to the growth medium of wild-type and knockout cells. 10 ng/ml of exogenous AprA significantly inhibited cell proliferation in both wild-type and *aprA*$^-$ cells. To further examine the function of AprA and due to limitations in purifying endogenous AprA for use in *in vitro* studies, I helped to make a recombinant form of AprA. Using recombinant AprA, I have characterized the bioactivity and binding kinetics of a secreted factor that binds with high affinity to live *Dictyostelium* cells.

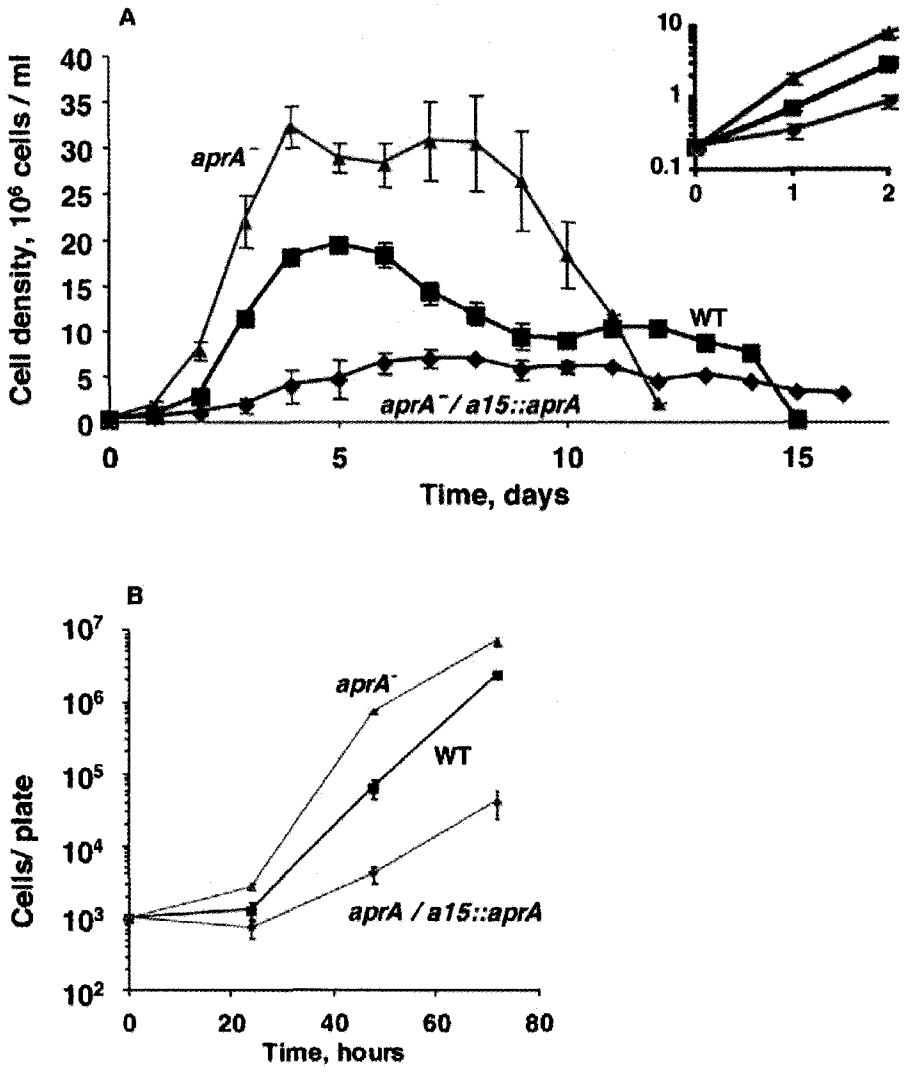

Figure 3. AprA slows the proliferation of cells. (A) Cell cultures were diluted to $2x10^5$ cells/ml in HL-5 medium, and the cell density was calculated daily. (B) The graph shows cell proliferation for each cell type on bacterial lawns on SM/5 plates (100 mm petri dishes). Day zero represents the initial starting value of 1000 cells for each cell line; *Dictyostelium* cells were counted at 24h, 48h, and 72h intervals. Between the 24 and 48 hour timepoints, the doubling times were 4.3 hours for wild-type cells, 3.0 hours for *aprA*$^-$ cells and 9.7 hours for *aprA*$^-$*/actin15::aprA* cells. The graphs show means ± SEM from three independent experiments (Brock and Gomer, 2005).

Cell type	Cell density after 24 hours ($\times 10^4$ cells/ml)	
	Control	10 ng/ml AprA
Wild type	31±2	21±2
aprA$^-$	53±4	25±3

Figure 4. Exogenous immunoprecipitated AprA inhibits cell proliferation. Cells were inoculated at $1x10^5$ cells/ml in shaking culture, and immunoprecipitated AprA or an equal volume of the material immunoprecipitated AprA from *aprA*$^-$ cells (control) was added. The cell density was then determined 24 hours later (Brock and Gomer, 2005).

Materials and Methods

Cell Culture

Cell culture was done following Brock and Gomer (1999) using wild-type (Ax-2) cells, *cfaD*$^-$ cells (unpublished), *aprA*$^-$ cells (Brock and Gomer, 2005), and *crlA*$^-$ (strain JH557) cells (Raisley et al., 2004). Conditioned growth medium (CM) was prepared and concentrated following Brock et al. (2002). Proliferation assays and calculation of doubling times were done as described in Brock and Gomer (2005).

Recombinant AprA Expression and Purification

An AprA cDNA was cloned as a EcoRI-XhoI fragment in pBAD/gIII(A) vector (Invitrogen) to express the recombinant AprA fused to a myc and His tag at its C terminus (pBAD-AprA). The plasmid pBAD-AprA was transformed into Top-10 *E. coli* competent cells (Invitrogen, USA) for rAprA expression, which was induced by adding 20% arabinose to a final concentration of 0.1% to the growing *E. coli* culture. After five hours of induction, the bacteria were collected by centrifugation and resuspended in PBS (10 mM KH_2PO_4, 150 mM NaCl, 2 mM KCl, pH 7.4) with EDTA-free protease inhibitors (Roche, USA). Cells were disrupted using a cell disruptor (EmulsiFlex-C5). N-lauroylsarcosine sodium salt solution (Fluka BioChemika, USA) was added to a final concentration of 5% to extract recombinant AprA into the supernatant. rAprA was purified using nickel agarose beads (Qiagen, USA) following the manufacturer's protocol.

Recombinant AprA Inhibition Assay

To test the bioactivity (cell proliferation inhibition activity) of rAprA and to determine whether CrlA and/or CfaD may be required for AprA proliferation inhibition activity, cells were incubated with varying concentrations of rAprA. Wild-type (Ax2), *aprA*$^-$, *crlA*$^-$, and *cfaD*$^-$ *Dictyostelium* cells were analyzed. All of the cell cultures ($5x10^5$ cells/ml) were incubated with (0, 0.01, 0.05, 0.1, 0.3, 1, 3, 10, 15, 30, or 60 μg/ml) of exogenous rAprA. Cells were counted before and after 12 hours of incubation with or without addition of rAprA.

With the possible involvement of CrlA, a 7-transmembrane, G-protein coupled receptor, we wanted to test the cell proliferation inhibition activity of rAprA to the G-alpha 1, 3, 5, 7, 8, and 9 mutant cell lines. AprA was added at a concentration of 1 μg/ml to the growth media of the G-alpha mutant cells. All of the cultures were started at a concentration of $5.0x10^5$ cells/ml. 1 μg/ml of rAprA was also added to wild-type cells to serve as a control. The cultures were shaken for 24 hours with cell counts taken after the 24 hour incubation period.

Binding Assays

Determination of optimal time to bind rAprA

To determine the saturation binding time of rAprA, wild-type (Ax-2) cells were grown to a density of 2-3 million cells/ml. Cells were collected by centrifugation, resuspended to a final concentration of 5.0 x 10^6 cells/ml and kept on ice. The cells were briefly washed twice and resuspended in ice cold HL-5 medium. Cells were then allowed to incubate with a fixed concentration of myc-tagged recombinant AprA (300 ng/ml) at 4°C on an end to end tumbler for 0, 1, 2, 5, 10, and 30 minutes. After the indicated times, cells were collected by centrifugation and washed briefly in ice cold HL-5. Following the wash, the cells were resuspended in SDS sample buffer (30 mL glycerol, 15 mL 1 M Tris pH 6.8, 3 mL BME, 6 g SDS) and heated before loading onto a pre-cast Tris-glycine gel (BioRad). Protein was transferred onto a PVDF membrane (Immobilin-P, Millipore, USA). Western blots were stained with anti-myc antibodies using different concentrations of myc-AprA as a standard. To determine the optimum time point for binding, signals were scanned and quantified using Image J and Microsoft Excel. Similar procedures were carried out on *aprA*$^-$, *crlA*$^-$, and *cfaD*$^-$ cells to determine the time of saturation of rAprA binding to the different cell lines.

Kinetic analysis

After determining the time needed to get nearly steady-state binding, varying amounts of rAprA (0, 20, 40, 80, 120, 160, 200, 400, 800, 1600 ng) were added to each of the four (wild-type (Ax-2), *aprA*$^-$, *crlA*$^-$, and *cfaD*$^-$) *Dictyostelium* cell lines to construct a binding curve for rAprA. The cells were prepared as mentioned above but incubated with different concentrations of rAprA for 10 minutes. After binding, the cells were collected by centrifugation and washed briefly in ice cold HL-5. Quantification was carried out as mentioned as above and information from the rAprA binding curve (using Graphpad Prism) was used to calculate the binding constant (K_D) of rAprA to cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, CA). Differences between two groups were assessed by Mann-Whitney or between multiple groups by ANOVA (Kruskal-Wallis) using Dunn's post-test. Significance was defined as $p < 0.05$.

Results

Recombinant AprA is bioactive

AprA is a secreted signal in *Dictyostelium* cells that regulates cell proliferation (Brock and Gomer, 2005). However, due to limitations in purifying large amounts of secreted endogenous AprA to study its function, we expressed and purified a recombinant version of the protein (Figure 5). To test the biological property of rAprA, we added varying amounts of rAprA to wild-type (Ax2), *aprA*$^-$, *crlA*$^-$, and *cfaD*$^-$ cells. Wild-type and *aprA*$^-$ cells proliferated 29% and 32%, respectively, more slowly compared to cells with addition of buffer alone at concentrations above 1 μg/ml of rAprA (Figures 6 and 7). But *crlA*$^-$ and *cfaD*$^-$ cells showed only ~3% less proliferation in presence of rAprA when compared to the buffer control (Figures 8 and 9). These results suggest that rAprA is bioactive and also indicate that CrlA and CfaD may be required for the biological function of the AprA.

Dictyostelium discoideum uses G-protein signal transduction for many developmental functions such as in cAMP signaling when the cells are starved for nutrients (Raisley, et al, 2004). To determine whether any of the G-protein subunits are involved in the AprA signal transduction pathway, rAprA inhibition assays were also performed on the G-alpha mutant cell lines. Cell proliferation was inhibited by about 16% in the wild-type cells, while rAprA inhibited cell proliferation by 8% for G-alpha 1, 12% for G-alpha 3, 4% for G-alpha 5, 12% for G-alpha 7, 1% for G-alpha 8, and close to 0% for G-alpha 9 (Figure 10). These results suggest that the G-alpha 8 and G-alpha 9

subunits are required for AprA cell proliferation inhibition activity and could both be involved in the AprA signal transduction pathway.

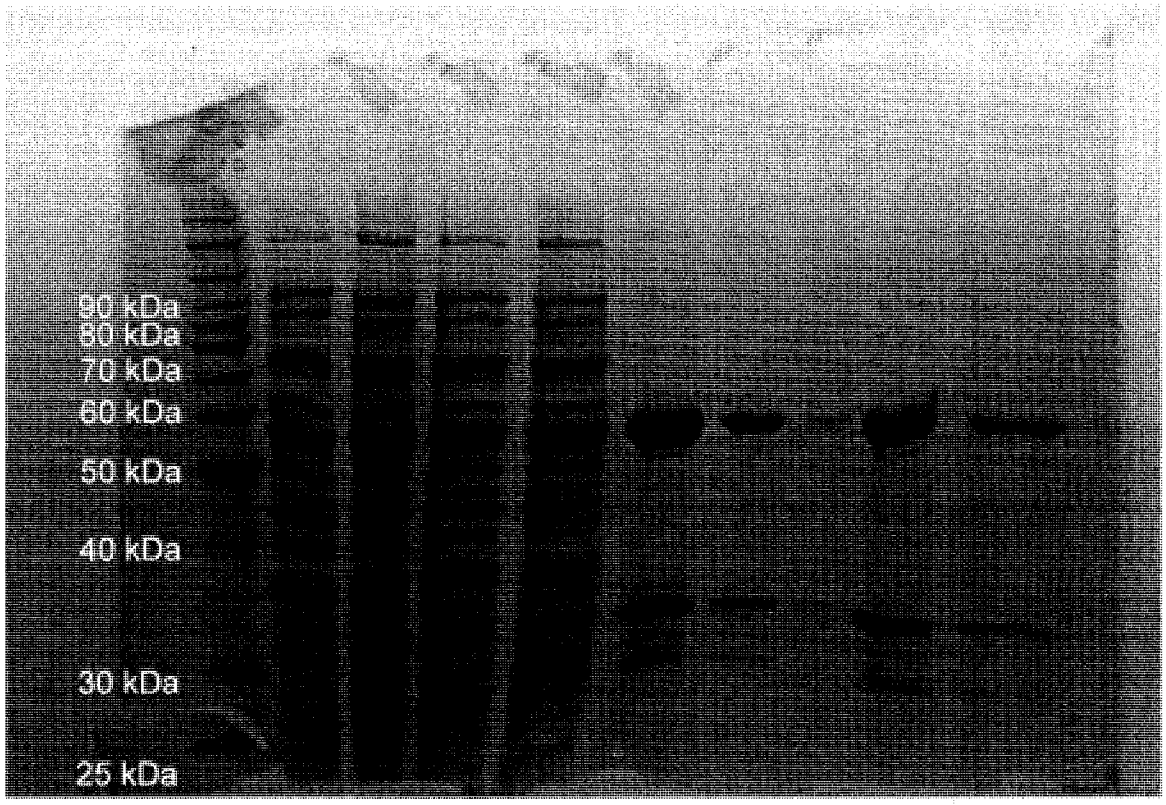

Figure 5: Recombinant AprA elutes at 60 kDa. AprA was expressed using a pBAD/gIII(A) vector transformed into electrocompetent *E. coli* cells. rAprA expression was induced with arabinose. rAprA was purified using nickel agarose beads. The molecular weight marker is shown to the left.

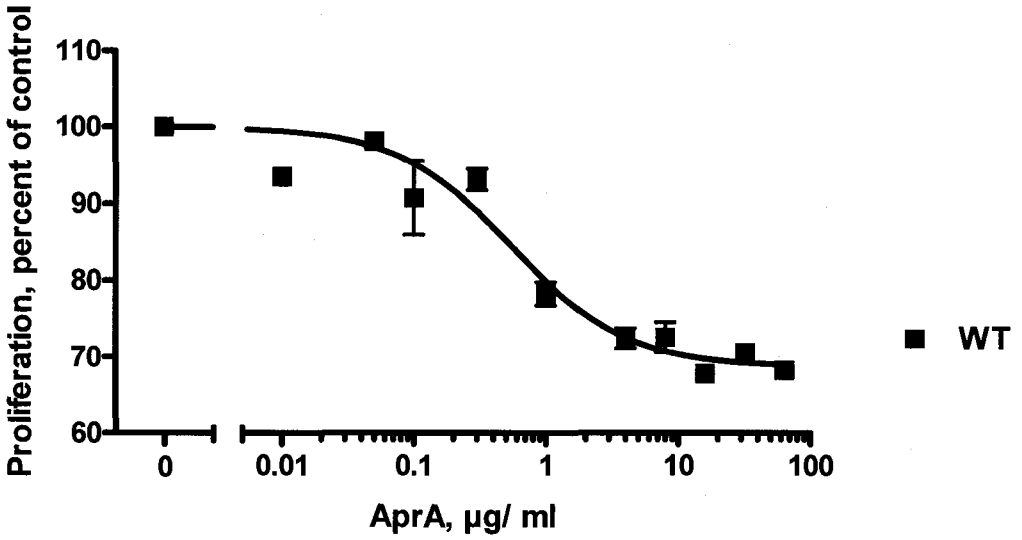

Figure 6. Exogenous rAprA slows the proliferation of wild-type cells. 5.0 x 10^5 wild-type cells in 1 ml HL-5 cultures were incubated with varying concentrations of recombinant AprA and allowed to grow for 12 hours. The proliferation at 12 hours was calculated as the cell density of cells treated with rAprA as a percent of the cells untreated with rAprA. Values represent the mean ± SEM for three independent experiments. The absence of an error bar indicates that the error bars were smaller than the plot symbol.

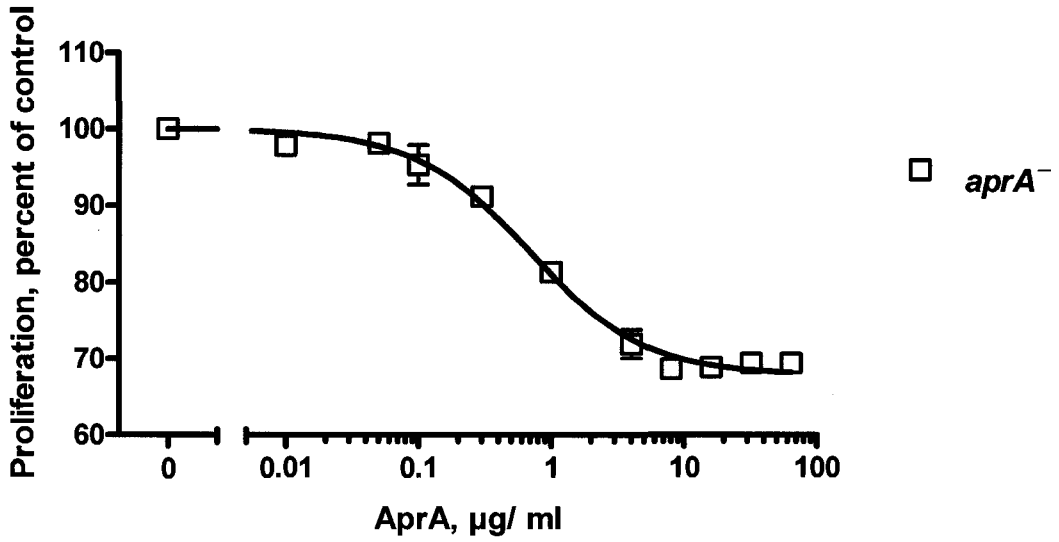

Figure 7. Exogenous rAprA slows the proliferation of *aprA*⁻ cells. 5.0 x 10^5 *aprA*⁻ cells in 1 ml HL-5 cultures were incubated with varying concentrations of recombinant AprA and allowed to grow for 12 hours. The proliferation at 12 hours was calculated as the cell density of cells treated with rAprA as a percent of the cells untreated with rAprA. Values represent the mean ± SEM for three independent experiments. The absence of an error bar indicates that the error bars were smaller than the plot symbol.

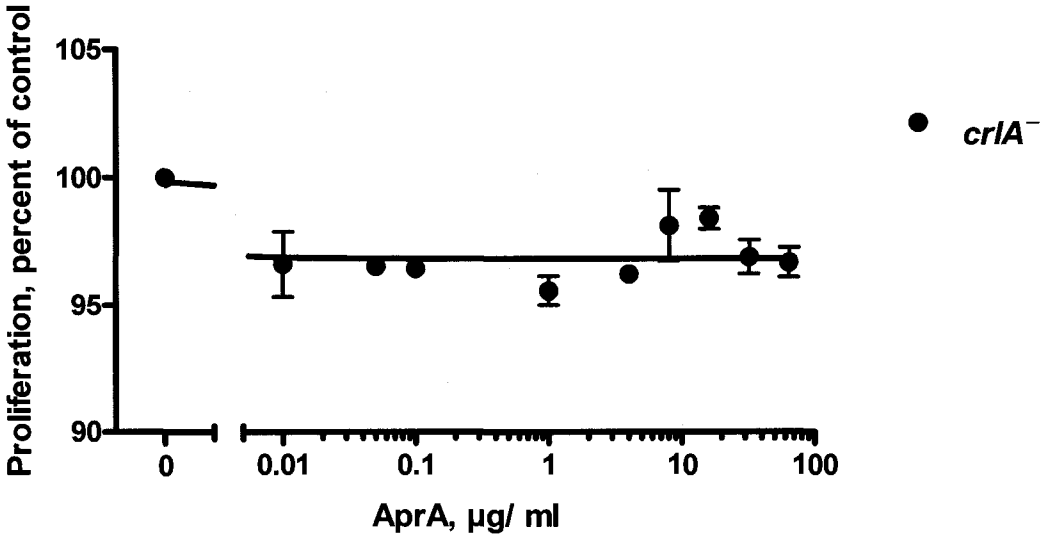

Figure 8. Exogenous rAprA does not slow the proliferation of *crlA*⁻ cells. 5.0 x 10^5 *crlA*⁻ cells in 1 ml HL-5 cultures were incubated with varying concentrations of recombinant AprA and allowed to grow for 12 hours. The proliferation at 12 hours was calculated as the cell density of cells treated with rAprA as a percent of the cells untreated with rAprA. Values represent the mean ± SEM for three independent experiments. The absence of an error bar indicates that the error bars were smaller than the plot symbol.

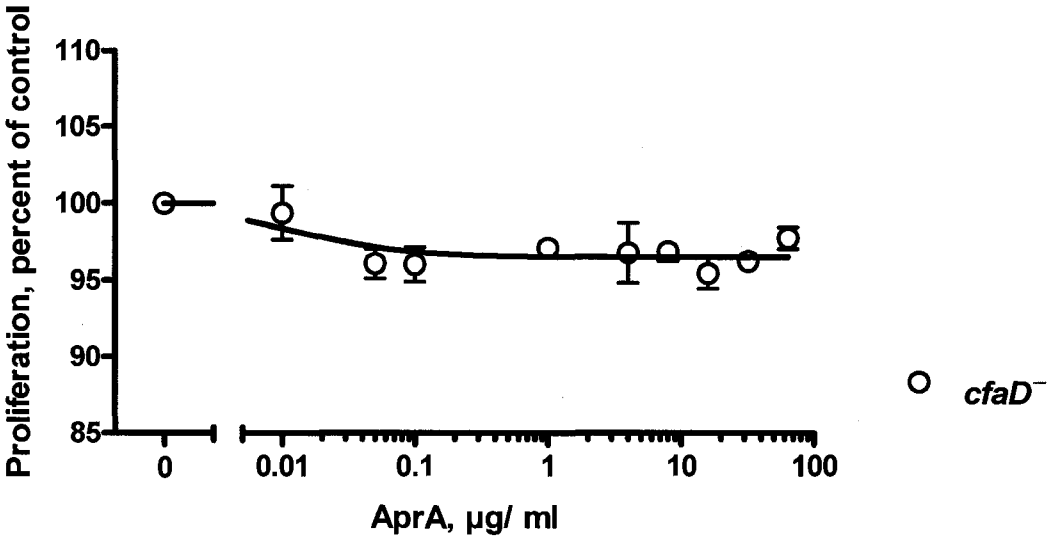

Figure 9. Exogenous rAprA does not slow the proliferation of *cfaD*⁻ cells. 5.0 x 10^5 *cfaD*⁻ cells in 1 ml HL-5 cultures were incubated with varying concentrations of recombinant AprA and allowed to grow for 12 hours. The proliferation at 12 hours was calculated as the cell density of cells treated with rAprA as a percent of the cells untreated with rAprA. Values represent the mean ± SEM for three independent experiments. The absence of an error bar indicates that the error bars were smaller than the plot symbol.

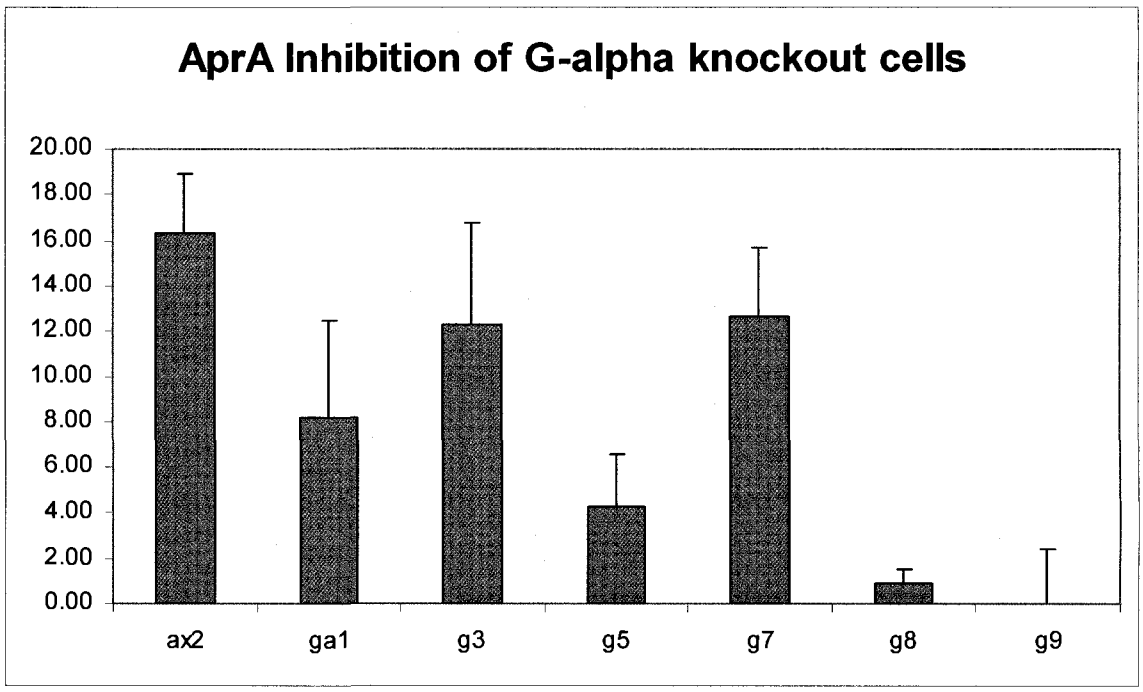

Figure 10. G-alpha 8 and G-alpha 9 subunits are required for AprA cell proliferation inhibition activity. Wild-type and G-alpha mutant cells were incubated with 1 μg/ml of recombinant AprA and counted after 24 hours. Values represent mean percentage of inhibition of growth rates compared to no AprA addition controls ± S.D. for six separate experiments.

Recombinant AprA binds to live *Dictyostelium* cells

Knowing that rAprA acts as a proliferation repressor, we wanted to investigate if rAprA bound to the cell surface of living *Dictyostelium* cells to activate the proliferation inhibition signal transduction pathway.

To establish the steady-state conditions for the binding assays, we examined the optimal time needed for rAprA to bind to live, intact cells. We incubated five million *aprA*$^-$ cells with 300 ng of rAprA for varying time periods. Figure 11 shows that rAprA bound instantly to *aprA*$^-$ cells, reaching saturation by 10 minutes and continued to bind a constant amount of rAprA for 30 minutes. Therefore, we considered doing all of the binding assays for 10 minutes. We found that *cfaD*$^-$ cells bound more rAprA than *aprA*$^-$ and wild-type cells (Figure 11). Statistical analysis (Paired t-test) also clearly indicated that the increased binding of rAprA by *aprA*$^-$ and *cfaD*$^-$ cells was significant ($p< 0.03$) in comparison to wild-type cells. However, even though Figure 11 suggested that *crlA*$^-$ cells bound more rAprA than wild-type cells, the differences were not statistically significant ($p > 0.09$).

We then analyzed if the binding was cooperative or non-cooperative by incubating wild-type cells with different concentrations of rAprA for a fixed time period of 10 minutes. Based upon this data, rAprA showed binding saturation at concentrations above 160 ng (Figure 12). Information from this binding curve was used to calculate a K_D of 157 ng/ml for wild-type cells. Fits of the data to binding equations with available Hill coefficient suggested that there was no binding cooperativity.

After determining the rAprA binding characteristics for wild-type cells, similar binding studies were carried out for rAprA binding to *aprA*$^-$, *crlA*$^-$, and *cfaD*$^-$ cells. Time course studies for these cell lines also showed a saturation binding time of 10 minutes (Figure 11). After determining the saturation binding time, rAprA binding curves were also constructed for the *aprA*$^-$, *crlA*$^-$, and *cfaD*$^-$ cell lines. Like the binding curve data for wild-type cells, rAprA showed maximal binding at concentrations above 160 ng (Figure 12). However, the dissociation constants differed between the mutant cell lines. Based on the binding curve data, the K_D for *aprA*$^-$ cells was 30 ng/ml, the K_D for *crlA*$^-$ cells was 114 ng/ml, while the K_D for *cfaD*$^-$ cells was 44 ng/ml. As for wild-type cells, there was no apparent binding cooperativity.

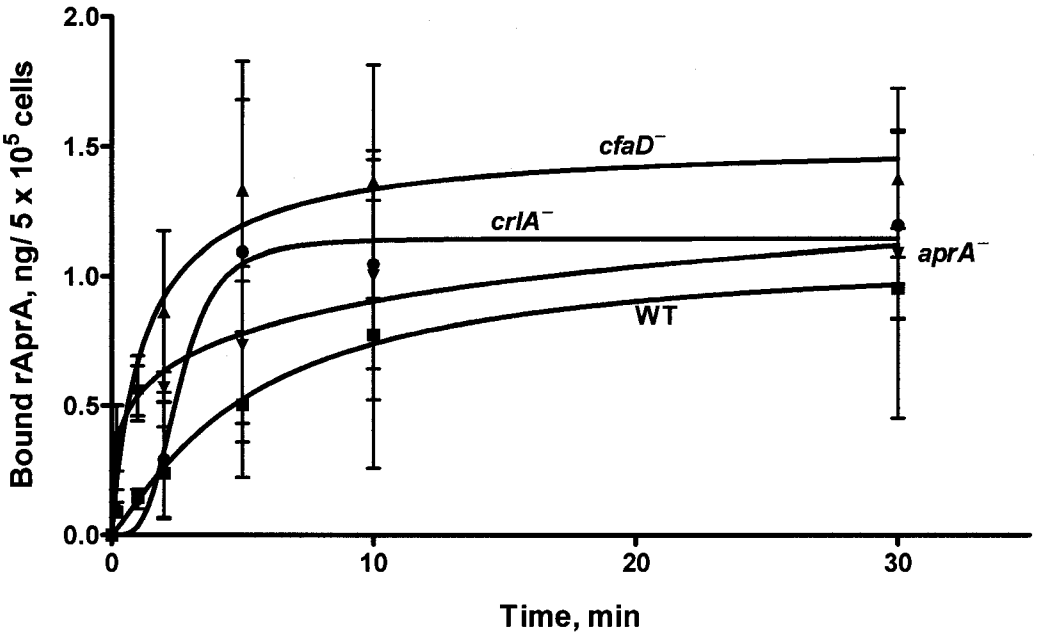

Figure 11. rAprA saturates receptors after 10 minutes of binding. A time course analysis was performed by allowing *Dictyostelium* cells (WT, *aprA⁻*, *crlA⁻*, *cfaD⁻*) to bind to a fixed concentration (300 ng/ml) of rAprA. The time points that were measured were for 0, 1, 2, 5, 10, and 30 minutes of binding. The bound rAprA was analyzed by western blots that also had different known concentrations of rAprA used for use as standards.

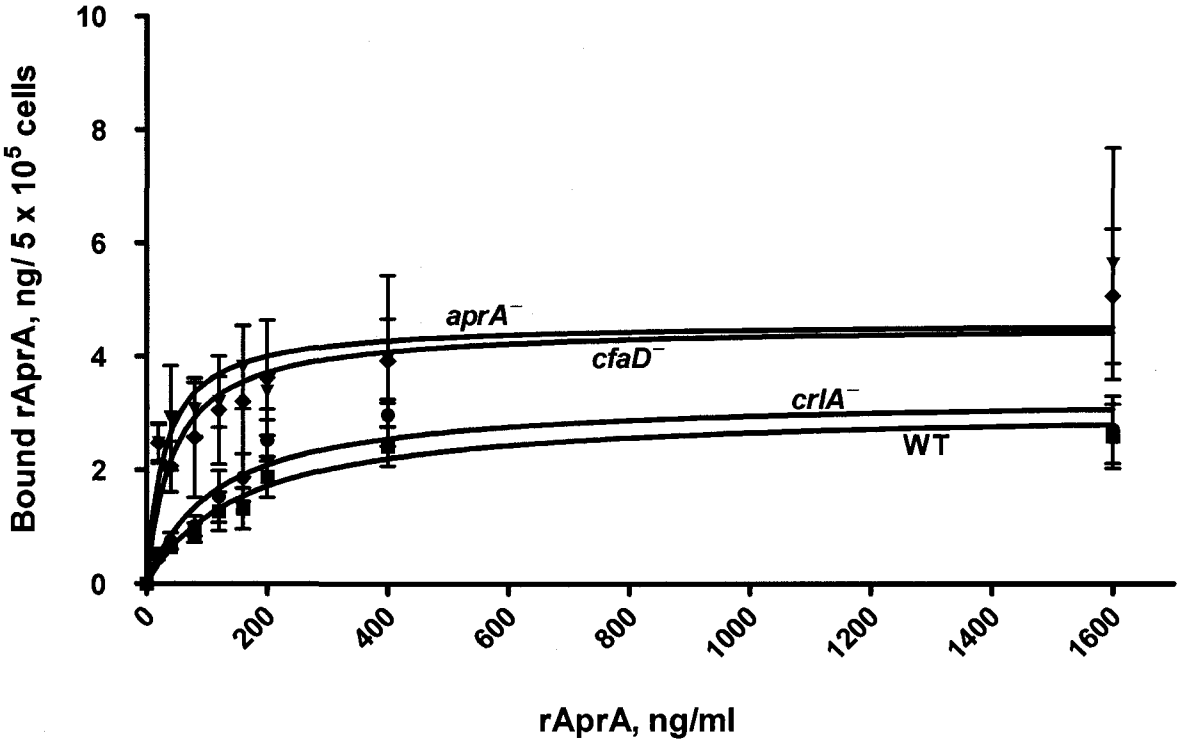

Figure 12. rAprA exhibits steady-state binding. A saturation curve analysis was performed by allowing *Dictyostelium* cells (WT, *aprA*⁻, *crlA*⁻, *cfaD*⁻) to bind to varying concentrations (0, 20, 40, 80, 120, 160, 200, 400, 800, 1600 ng/ml) of rAprA. rAprA was allowed to bind to the cells for 10 minutes at 4°C. The bound rAprA was analyzed by western blots that also had different known concentrations of rAprA for use as standards. Values are mean ± SEM from 3 independent experiments.

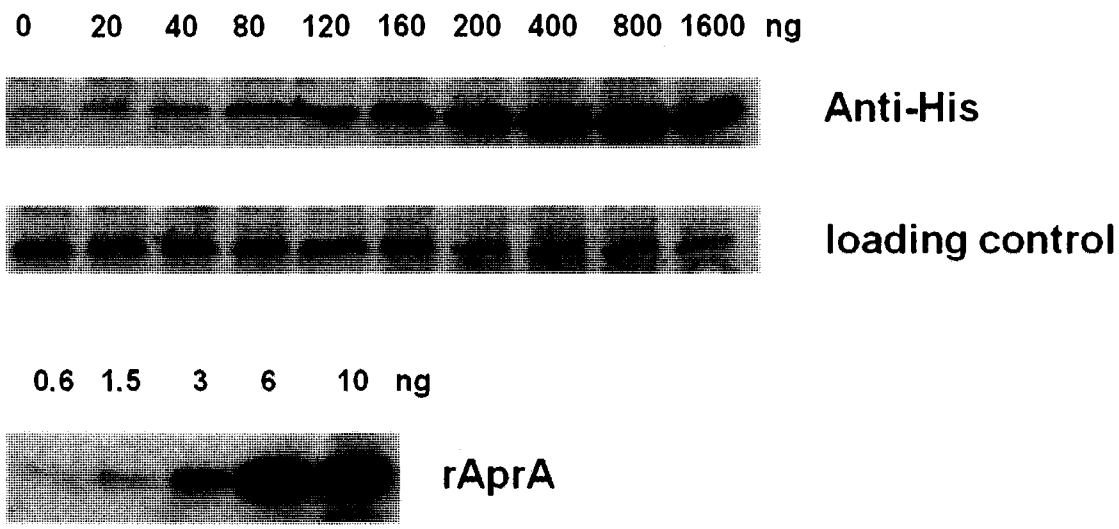

Figure 13. rAprA exhibits saturable binding to *Dictyostelium* cells. The top panel shows a western blot stained with anti-His antibody of total cell lysate that has taken up varying amounts of rAprA at different concentrations for a constant time period of 10 minutes. The middle panel shows that there was equal loading of protein samples for each concentration point. The bottom panel shows the rAprA standards that were run on the same gel and used to calculate the concentration of bound rAprA. These images are representative of at least three independent experiments.

Discussion

Dictyostelium cells appear to regulate cell proliferation by secreting and sensing AprA. In cells that do not secrete AprA, cell proliferation is unchecked as cells grow to a very high density in a short period of time as compared to cells that secrete a steady amount of AprA (Brock and Gomer, 2005). To further examine the inhibitory nature of AprA, we purified a bacterially synthesized AprA (recombinant AprA). We have found that rAprA has cell proliferation inhibition activity and binds to cells with relatively high affinity to negatively regulate cell proliferation.

Assays with wild-type and *aprA*$^-$ cells treated with immunoprecipitated AprA showed that adding exogenous AprA inhibited cell proliferation (Brock and Gomer, 2005). Our data supports this finding as the addition of exogenous recombinant AprA at concentrations as low as 0.1 μg/ml inhibited cell proliferation by about 15% to a high of 30% beyond 1 μg/ml in comparisons to controls without the addition of AprA in both wild-type and *aprA*$^-$ cells. However, we need to understand how the AprA signal is sensed by the cells. To try to understand this, we looked for cell surface receptor mutants with similar phenotype as cells lacking AprA. Of the existing receptor mutants, cells lacking CrlA proliferate faster than wild-type cells (Raisley et al., 2004). Interestingly, *crlA*$^-$ cells did not respond to the rAprA inhibitory signal. Meanwhile, we had identified another secreted factor, CfaD, which also behaved like AprA (unpublished data). Like *crlA*$^-$ cells, *cfaD*$^-$ cells were also insensitive to the rAprA signal. All together, the data initially suggested that CrlA may be the receptor for AprA while CfaD could be a cofactor that helps AprA in the process of inhibiting cell proliferation.

To examine the binding properties or rAprA to cell surface receptors, we performed ligand binding assays using rAprA to bind to live, intact *Dictyostelium* cells. We found a relatively fast association of rAprA to cell surface receptors. Wild-type cells showed maximal levels of binding after 10 minutes of incubation, which is similar to the binding kinetics observed for another secreted factor, recombinant countin (Gao et al., 2002). This fast association would make cells more sensitive to large increases in AprA concentrations, which might increase the rate of receptor endocytosis (Planque, 2006). In addition to the time of maximal rAprA binding, we found saturable rAprA binding at approximately 60,000 binding sites/cell with a K_D of 157 ng/ml, which is similar to the binding kinetics of conditioned medium factor (Jain and Gomer, 1994). This binding is very specific as we found that rAprA bound to wild-type cells in the presence of bovine serum albumin and recombinant serum amyloid P (unpublished data). The recombinant AprA binding can be competed off by the addition of native AprA present in wild-type conditioned growth medium (preliminary data not shown).

The binding kinetics of rAprA to *crlA*$^-$ cells were similar to rAprA bound to wild-type cells as rAprA bound with a K_D of 114 ng/ml to *crlA*$^-$ cells. This observation strongly suggests that CrlA may not be the receptor to AprA. However, CrlA might still have a role in assisting AprA's cell proliferation inhibition as the CrlA knockout cells proliferate just as fast as the AprA knockout cells (Raisley et al., 2004). On the other hand, rAprA bound with more affinity to *cfaD*$^-$ cells (K_D of 44 ng/ml) and to *aprA*$^-$ cells (K_D of 30 ng/ml).

Although we still do not completely understand the AprA signal transduction pathway, we believe that CfaD is a key component of the pathway. Further biochemical analysis will be needed to determine whether CfaD acts downstream of AprA or whether both proteins interact together (like cofactors). While CrlA, a 7-transmembrane, G-protein coupled receptor, appears not to be the AprA receptor, other G-protein coupled receptors or G-protein subunits may be involved in the AprA signal transduction pathway. Preliminary data supports that G-alpha subunits 8 and 9 may be involved in the pathway. Further elucidation of the AprA signal transduction pathway will help us to understand how *Dictyostelium* cells repress their own proliferation and what ramifications this negative regulation might have in similar signal transduction pathways in higher eukaryotes.

Appendix

AprA^{-} cells have a greater percentage of cells in the S phase of the cell cycle

To further examine whether AprA and CrlA regulate cell proliferation via a common pathway, BrdU staining experiments were conducted to calculate the percentage of cells actively replicating in the S phase of the cell cycle. BrdU cell staining was performed on *apr*A^{-}, *apr*A^{OE}, and wild-type *Dictyostelium* cells. 1 ml cultures of each strain was placed in Falcon tubes at a cell density of $5.0x10^5$ cells/ml. BrdU was added to the cells at a final concentration of 300 uM, and the tubes were placed on a shaker. After 2 hours, 500 µl of the cells were placed in a well of an eight-well slide. The cells were allowed to settle for 30 minutes and then fixed with 10% paraformaldehyde in PBS. The cells were then washed once with PBS/0.5% Tween. Anti-BrdU mAb was diluted 1:50 in nuclease buffer (Amersham) and added to the wells for one hour. The cells were washed with PBS/0.5% Tween, and secondary antibody (Alexa: fluorescein conjugated goat anti-mouse, 1:1000 dilution in PBS/1% BSA) was added to the wells for one hour. The wells were washed for a final time with PBS and then mounted with a coverslip and DAPI stain. I expected that a greater percentage of cells would be in the S phase for *apr*A^{-} as compared to wild-type because *aprA*$^{-}$ cells proliferate at a more rapid rate and would be more likely to have cells actively replicating in the S phase of the cell cycle. I also expected that AprA^{OE} cells would have a lesser percentage of cells in the S phase as compared to wild-type as the overexpressing cells have a slower growth rate.

From a previous study, approximately 10% of the *Dictyostelium* wild-type cells were in the S phase of the cell cycle (Wood et al., 1996). In my experiments, I found that approximately 21% of the *aprA*$^{-}$ cells, 16% of the wild-type cells, and 11% of AprA^{OE}

cells were in the S phase of the cell cycle (Figure 15). This work was performed before the rAprA binding experiments to *crlA*$^-$ cells. The rAprA binding experiments indicated that CrlA is probably not the receptor for AprA. Thus, I did not follow up on performing the BrdU staining experiments with CrlA knockout cells. However, the BrdU staining experiments will be performed with the G-alpha 8 and 9 mutant cell lines following previously stated procedures to determine whether the G-protein subunit mutants negatively regulate cell proliferation via a common pathway with AprA.

Figure 14: BrdU incorporation in actively replicating *Dictyostelium* cells. The green fluorescence indicate cells actively replicating in the S phase of the cell cycle while the blue represents nuclear DNA. This is a representative photo of wild-type cells.

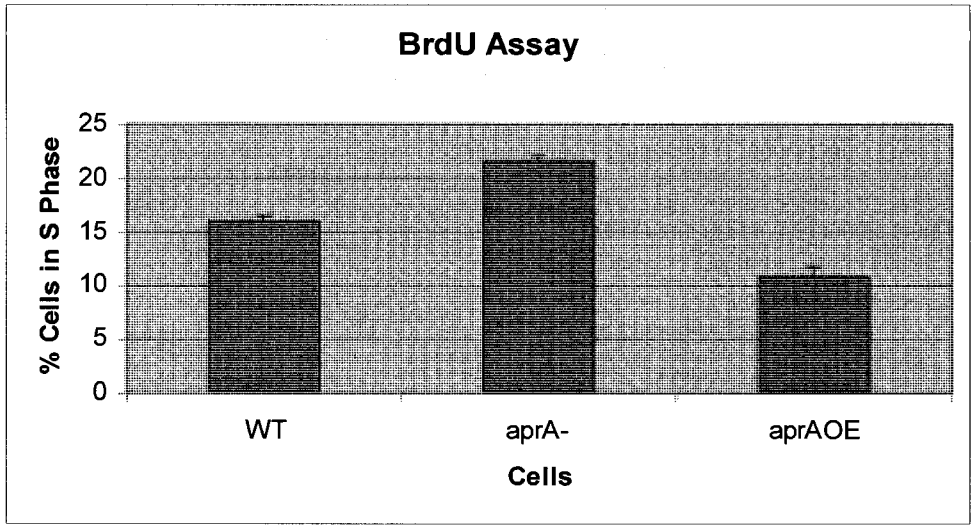

Figure 15: AprA knockout cells have a greater percentage of cells in the S phase of the cell cycle. Values are mean percentages +/- S.D. for six separate experiments.

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