RICE UNIVERSITY

Regulation of cell number and cell movement by autocrine signaling in

*Dictyostelium discoideum*

by

Jonathan Phillips

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APPROVED, THESIS COMMITTEE

Richard Gomer, Professor, Advisor
Biochemistry and Cell Biology

Kathleen Beckingham, Professor
Biochemistry and Cell Biology

Janet Braam, Chair and Professor
Biochemistry and Cell Biology

Cindy Farach-Carson, Vice Provost
for Translational Bioscience and Professor
Biochemistry and Cell Biology

Jeff Tabor, Assistant Professor
Bioengineering

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Abstract

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Little is known about how the size of a tissue is established during development and maintained subsequently. Proliferation-inhibiting signals secreted by cells within a tissue that act specifically on cells within that tissue can provide negative feedback on cell number, thus regulating tissue size. A better understanding of tissue-specific inhibitors of proliferation could be useful for designing therapies for cancer and other diseases. However, few signals of this sort have been identified, and little is known about how these signals function. Two examples of such signals are the proteins AprA and CfaD, which are secreted by the social amoeba *Dictyostelium discoideum* and inhibit cell proliferation in a concentration-dependent manner. Cells lacking either AprA or CfaD proliferate rapidly, and adding recombinant AprA or CfaD to cells reduces proliferation. However, little is known about the signal transduction pathways downstream of AprA and CfaD. I identified three proteins that are required for the normal function of AprA and CfaD: the kinase QkgA, the putative transcription factor BzpN, and the putative kinase PakD. Cells lacking any one of these proteins proliferate rapidly, and adding AprA or CfaD to cells lacking these proteins does not cause reduced proliferation, indicating that these proteins are involved in AprA/CfaD signal transduction. I also found that, in addition to its proliferation-inhibiting activity, AprA also functions as an
autocrine chemorepellant. Colonies of cells lacking AprA expand less rapidly than wild-type colonies, despite the fact that individual cells lacking AprA show a random motility like that of wild-type cells. Further, two independent assays demonstrate that cells show a biased movement away from a source of AprA. The chemorepellant activity of AprA requires CfaD, QkgA, and PakD, but not BzpN, indicating that AprA affects proliferation and chemorepulsion through distinct but overlapping pathways. These results suggest that AprA functions as a readout of local cell density, to which cells respond by slowing proliferation and chemotaxing to regions of lower cell density, where nutrients are more likely to be present. The study of human AprA, CfaD, QkgA, BzpN, and PakD orthologs may serve to guide therapeutic approaches that modulate chemorepulsive or antiproliferative processes.
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Chapter 1. Autocrine regulation of cell number in metazoans and in

*Dictyostelium discoideum*

**A mechanism for the regulation of organ size**

Despite the existence of sophisticated, experimentally supported explanations for the establishment and patterning of distinct tissues during organismal development, mechanisms underlying the regulation of organ size during development and homeostatic maintenance of organ size beyond organogenesis are poorly elucidated. Assuming that an organ is primarily composed of cells, such a mechanism would necessarily involve regulating some combination of cell size, proliferation, growth, and death so as to generate a tissue of a certain volume. Several lines of evidence suggest that, at least in some cases, this size regulation mechanism is intrinsic to the organ and may involve soluble factors. For instance, when spleens are removed from mice and a few small pieces of spleen are grafted in multiple sites in a recipient mouse, the grafts grow to a total mass equivalent to that of a normal spleen (Metcalf, 1964). Additionally, transplant experiments between closely related salamanders of considerably different size have shown that transplanted eyes or limbs reach the size expected of the donor, and not the recipient, supporting an organ-autonomous mechanism (Twitty and Schwind, 1931). Furthermore, the regenerative capacity of some tissues, such as the mammalian liver (Michalopoulos and DeFrances, 1997) and Drosophila imaginal discs (Bryant, 1975) indicate that organ size is, in some cases, actively monitored beyond initial establishment. In both of these tissues, the ablation of cells results in a mitotic burst (Bucher et al., 1951;
Smith-Bolton et al., 2009), suggesting that tissues can sense and respond to a decrease in tissue size.

Autocrine signals are signals produced by and acting on the same population of cells. An explanation for control of tissue size, postulated by William Bullough in 1962 (Bullough, 1962), suggests that anti-proliferative autocrine factors secreted by and acting upon individual tissues may regulate the size of an organ. In this theoretical mechanism, cells composing a tissue secrete the anti-proliferative factor at a constant rate and respond in a dose-dependent manner. To independently regulate the size of different tissues, each tissue secretes a factor that only represses the proliferation of cells specific to that tissue. At some concentration of the factor, the net mitotic rate is repressed to the point where a static tissue size is maintained. Candidate factors (heat labile tissue-local inhibitors of mitosis) have been characterized for liver, kidney (Saetren, 1956) and epidermal (Bullough et al., 1964) tissues, although the specific active factors have not been purified.

More recently, two true autocrine repressors of proliferation have been purified and characterized in vertebrates. The protein myostatin, a TGF-beta superfamily member, is secreted by skeletal muscle fibers and represses the proliferation of muscle cell progenitors (Thomas et al., 2000). Myostatin null mutant mice exhibit both hyperplasia and hypertrophy of skeletal muscle (McPherron et al., 1997). Similarly, GDF11, also a TGF-beta superfamily member, is expressed by olfactory epithelium neurons in mice and inhibits the proliferation of neuronal precursor cells. This negative feedback loop serves to regulate olfactory epithelium size (Wu et al., 2003). These
findings confirm that autocrine repression of proliferation regulates the size of some tissues and suggest that similar but uncharacterized mechanisms may exist in other tissues.

The secreted proteins AprA and CfaD slow the proliferation of cells in *Dictyostelium discoideum*

We have identified two proteins in the eukaryote *Dictyostelium discoideum* that, similar to the factors discussed previously, are secreted and sensed by a cell population and inhibit proliferation in a dose-dependent manner (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). *Dictyostelium* exists as a single-celled amoeba that lives in the soil and feeds upon bacteria. However, upon starvation, *Dictyostelium* cells cease dividing and begin to secrete and respond to the chemoattractant cyclic AMP (cAMP), leading to the streaming of cells toward sites of aggregation. These aggregates culminate in the development of a fruiting body, a multicellular structure composed of a thin column of stalk cells with a mass of spores at the top. Raising the spores off the soil allows spores to spread to more hospitable locations (Kessin, 2001). This developmental process will not proceed unless sufficient levels of the autocrine quorum-sensing signal CMF are present, serving to restrict development to conditions where enough cells to generate a complete fruiting body are nearby (Gomer et al., 1991). The segregation between the stages of proliferation (known as vegetative stage) and multicellular development provides a tractable system for the study of factors and signal transduction pathways involved in regulating these distinct processes. The powerful genetic tools of gene disruption by homologous recombination, overexpression, and gene silencing by antisense are
established for *Dictyostelium*, and the lack of a cell wall greatly facilitates biochemical and cell biological studies of the model organism.

While studying factors that regulate the size of fruiting bodies, our lab identified a gene encoding a secreted protein that, when knocked out, resulted in a rapid proliferation phenotype (Brock and Gomer, 2005). This protein was named AprA. AprA has little similarity to any human protein, though highly similar proteins are encoded in the genomes of the chordate amphioxus, the tunicate Ciona intestinalis, and the choanoflagellate Monosiga brevicollis. Growth curves of *aprA*− cells show that loss of AprA results in both a faster rate of proliferation and a higher stationary cell density as compared to wild type cells, whereas cells overexpressing AprA under control of the constitutive actin15 promoter proliferate more slowly and reach a lower maximum cell density (Figure 1 (Brock and Gomer, 2005)). *aprA*− cells show no difference in accumulation of mass or protein content per nucleus as compared to wild type, indicating that AprA inhibits proliferation, but not growth on a per nucleus basis (Brock and Gomer, 2005). However, as *aprA*− cells proliferate more rapidly than wild type, and thus accumulate nuclei more rapidly, an equivalent rate of growth per nucleus between wild-type and *aprA*− cells results in more mass and protein accumulation in a population of *aprA*− cells as compared to wild type. Thus AprA does affect mass and protein accumulation at the population level. Nuclear staining using the DNA-staining dye DAPI has revealed that *aprA*− cells are more often multinucleate than wild type, suggesting that cytokinesis may lag in respect to mitosis in *aprA*− cells. Furthermore, addition of immunoprecipitated or recombinant AprA (rAprA) to cells slows proliferation, indicating
Figure 1: AprA slows the proliferation of cells. Cells were diluted to 2x10^5 cells/ml in axenic media and the cell density was measured daily. The graph shows means±s.e.m. from three independent experiments. Figure adapted from Brock and Gomer (Brock and Gomer, 2005).
that AprA exerts its antiproliferative activity through an extracellular signaling mechanism.

A second protein, termed CfaD (for counting factor associated (Brock and Gomer, 1999)), exhibits properties like those of AprA. CfaD has homology to the conserved family of cathepsin-L proteases, although the protein shows no protease activity on established cathepsin substrates (Bakthavatsalam et al., 2008). \textit{cfaD} \textsuperscript{-} cells proliferate faster than wild type, and cell proliferation is slowed by the addition of recombinant CfaD (rCfaD). AprA from conditioned medium co-elutes on sieving gel chromatography with CfaD, and AprA co-immunoprecipitates with rCfaD, suggesting that AprA and CfaD associate with each other and are involved in the same proliferation-repressing mechanism. Additionally, rAprA does not inhibit the proliferation of \textit{cfaD} \textsuperscript{-} cells, and rCfaD does not inhibit the proliferation of \textit{aprA} \textsuperscript{-} cells, indicating a requirement for both proteins in proliferation inhibition (Bakthavatsalam et al., 2008).

Although little is known about signal transduction downstream of AprA and CfaD, heterotrimeric G protein complexes have been implicated in AprA signaling (Bakthavatsalam et al., 2009). G protein complexes are composed of \( \alpha \), \( \beta \), and \( \gamma \) subunits that dissociate upon binding of GTP to the \( \alpha \) subunit in response to some stimulus (generally a ligand binding to a G protein-coupled receptor). The dissociated subunits then activate downstream components of the signal transduction pathway (McCudden et al., 2005). The addition of recombinant AprA to purified cell membranes increases binding of GTP to the membranes, suggesting that G proteins function in AprA signaling.
(Bakthavatsalam et al., 2009). Of the 9 known α subunits encoded in the *Dictyostelium* genome, Gα8 and Gα9 null mutants, but not other G protein null mutants, show faster proliferation than wild type and are insensitive to proliferation inhibition by rAprA. However, the addition of recombinant AprA increases binding of GTP to gα9− cell membranes but not gα8− membranes, suggesting that AprA activates a proliferation-inhibiting signal transduction pathway of which Gα8 but not Gα9 is a component (Bakthavatsalam et al., 2009). These results suggest that AprA signals through Gα8 and that a parallel but linked pathway negatively regulates proliferation and involves Gα9. Downstream components of AprA and CfaD-mediated signaling beyond Gα8 are not known, although cells mutant for the kinase Quick growth A (QkgA), show a rapid proliferation phenotype corresponding to that of aprA− cells (Abe et al., 2003).

Many *Dictyostelium* signal transduction pathways are conserved in humans, though the extracellular signals that mediate signaling tend to vary. For instance, in *Dictyostelium* and in human neutrophils, cellular chemotaxis is mediated by a signaling cascade involving PI3 kinase and Akt (Meili et al., 1999; Servant et al., 2000), though cyclic AMP serves as the primary chemoattractant in *Dictyostelium* (Konijn et al., 1968), and bacterially produced N-formyl methionine (among other signals) is a chemoattractant in neutrophils (Schiffmann et al., 1975). Additionally, both human glycogen synthase kinase 3 (GSK3) and its *Dictyostelium* orthologue GskA function in cell fate specification (Harwood et al., 1995), though this process is mediated by extracellular cAMP in *Dictyostelium* and members of the wnt protein family in humans (Kim and Kimmel, 2000). Therefore, understanding how the secreted proteins AprA and CfaD function
together to regulate cell proliferation in *Dictyostelium*, and in particular understanding the signal transduction pathways involved, may reveal conserved proliferation-regulating mechanisms that could serve to control tissue size.
Chapter 2. The ROCO kinase QkgA is required for proliferation inhibition by AprA and CfaD

Introduction

Like aprA− and cfaD− cells, Dictyostelium cells lacking the ROCO family kinase QkgA show abnormally rapid proliferation (Abe et al., 2003). The ROCO protein family is widely conserved and is defined by the presence of a Ras of complex proteins (Roc) domain followed by a C-terminal of Roc (Cor) domain, which mediates homodimerization (Gotthardt et al., 2008). In eukaryotes, these domains are commonly followed C-terminally by a kinase domain with similarity to the tyrosine kinase-like (TKL) group of kinases (Anand and Braithwaite, 2009; Lewis, 2009; Marin et al., 2008). In Dictyostelium, other ROCO proteins function in cyclic GMP signaling (Bosgraaf et al., 2005; van Egmond et al., 2008) and cytokinesis (Abysalh et al., 2003), and a total of 11 predicted ROCO proteins are present in the Dictyostelium discoideum genome, 10 of which, including QkgA, encode kinase domains predicted to be catalytically active (Goldberg et al., 2006). The human genome encodes two ROCO kinases, which are expressed in a wide range of tissues (Korr et al., 2006; Zimprich et al., 2004). Little is known regarding the physiological function of these proteins, although the ROCO protein LRRK2 is implicated in a dominantly inherited form of Parkinson’s disease (Zimprich et al., 2004) and negatively regulates neurite growth in rat cortical cultures (MacLeod et al., 2006).

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1 The work in this chapter has been published (Phillips and Gomer, 2010).
I investigated whether QkgA is involved in AprA/CfaD signaling and found that, like \textit{aprA}^- and \textit{cfaD}^- cells, \textit{qkgA}^- cells proliferate to a higher cell density than wild type and tend to be multinucleate. Additionally, I found that \textit{qkgA}^- cells are insensitive to exogenous AprA and CfaD, indicating that QkgA is required for AprA and CfaD signal transduction.

**Materials and Methods**

**Cell culture, immunoblotting, AprA/CfaD inhibition assays, and spore viability**

\textit{qkgA}^- cells (Abe et al., 2003) were a kind gift from Dr. Jeff Williams. Ax2 wild-type, \textit{qkgA}^- clone DBS0236839 (Abe et al., 2003), \textit{aprA}^- clone DBS0235509 (Brock and Gomer, 2005), and \textit{cfaD}^- clone DBS0302444 (Bakthavatsalam et al., 2008) cells were grown in HL5 media (Formedium Ltd, Norwich, England) as previously described (Brock and Gomer, 1999). To generate cells expressing the QkgA C-terminal GFP fusion, cells were transformed with the extrachromosomal vector QkgA-pDM323 (van Egmond and van Haastert, 2010) and selected in 15 \(\mu\)g/mL geneticin. For each transformation, at least two clones were analyzed and had a similar proliferative phenotype. For proliferation curve experiments, all cells were grown in the absence of selective drugs. To compare levels of extracellular AprA and CfaD, cells in axenic shaking culture at 3 \(\times\) \(10^6\) cells/mL or 12 \(\times\) \(10^6\) cells/mL were collected by centrifugation at 3000 \(\times\) g, and a sample of the supernatant was boiled in an equivalent volume of 2X loading buffer. 20 \(\mu\)l of these samples were run on a 4-15\% polyacrylamide gel and Western blots were stained for AprA following (Brock and Gomer, 2005) or for CfaD following (Bakthavatsalam et al., 2008). Proliferation inhibition assays were done
following (Choe et al., 2009), with an incubation time of 16 hours. As considerable variation in cell density was observed between separate experiments, all proliferation inhibition data is shown as a percent inhibition of proliferation (that is, percent difference in cell density) as compared to a paired control condition in which only buffer has been added to cells. For spore viability assays, $10^7$ cells were washed once in 8 mL PDF (20 mM KCl, 9.2 mM K$_2$HPO$_4$, 13.2 mM KH$_2$PO$_4$, 1mM CaCl$_2$, 2.5 mM MgSO$_4$, pH 6.4), resuspended in 750 µl PDF, deposited on halved black filter pads (8 micron pore size, 47 mm diameter, Millipore) on a pad of Whatman No. 3 filter paper soaked in PDF, and allowed to develop for 48 hours. Filters were placed in an Eppendorf tube containing 1 mL PDF + 0.5% Triton X-100 and briefly vortexed to disaggregate fruiting bodies. The filter was then removed and cells were incubated in PDF + 0.5% Triton X-100 for five minutes. Spores were collected by centrifugation at 6000 x g for 1 minute, resuspended in 1 mL PDF, and counted by hemocytometer. After dilution in PDF, 50 spores were plated in triplicate on SM/5 plates with K. aerogenes bacteria, and the number of resultant plaques was counted after four days to assess the number of viable spores. For measurement of colony diameter, cells growing in HL5 media at densities of 1-3 x $10^6$ cells/mL were serially diluted, mixed with K. aerogenes bacteria, and spread on SM/5 plates. Plates with well-spaced colonies were imaged daily, and the diameter of at least 3 colonies per genotype per day was measured using ImageJ software (Sheffield, 2007).

**Mass and protein determination**

To determine the mass of $10^7$ cells, 5 x $10^7$ cells from shaking cultures in HL5 media at densities of 2-5 x $10^6$ cells/mL were collected by centrifugation for 3 minutes at
1500 x g, and the pellet was resuspended in approximately 1 mL of residual media and transferred to a microcentrifuge tube. After centrifugation for 3 minutes at 3000 x g, the supernatant was removed, the cell pellets were weighed, and the value was divided by five. To determine the protein content of cells, the pellets were then resuspended to a total volume of 1 mL in PBM [20 mM KH$_2$PO$_4$, 0.01 mM CaCl$_2$, 1 mM MgCl$_2$ (pH 6.1) with KOH] and were frozen at -80°C. Following thawing, cells were resuspended by vortexing and 100 µl of the lysed cells was mixed with 100 µl of PBM containing 0.2% SDS and 0.2% Triton-100. Lysates were rotated at room temperature for 10 minutes and protein concentrations were measured with a Bio-Rad protein assay (Hercules, CA) by combining 20 µl of lysate sample with 1 mL of 1x Bio-Rad dye. Solutions of 1.0, 0.5, 0.25, and 0.125 mg/mL BSA in buffer equivalent to the lysates were used as standards.

**Calculation of doubling times**

To calculate the doubling time $t_d$ of cell cultures during exponential growth, the following equation was used:

$$t_d = \frac{\ln (2)}{\ln \left(\frac{P_n}{P_0}\right) / t}$$

where $P_0$ is the initial cell density, $P_n$ is the final cell density, and $t$ is the time interval in hours. Doubling times were calculated during growth from day 1 to day 5 from cultures inoculated at 1x10$^5$ cells/mL.

**Fluorescence microscopy**

DAPI staining and imaging of nuclei were done as described previously (Brock and Gomer, 2005). For imaging of cells expressing QkgA-GFP, cells growing in HL5
were washed and resuspended in low-fluorescence axenic media (Bokko et al., 2007),
grown overnight in shaking culture, collected by centrifugation at 1500 x g, and
resuspended in low-fluorescence media at a volume equivalent to the starting volume. A
drop of cells was placed on a glass slide and imaged with a Zeiss Axioplan II microscope.
For deconvolution imaging, cells growing in shaking culture in HL5 at densities of 1-4 x
10^6 cells/mL were allowed to settle on a glass coverslip for ten minutes, fixed in 4%
paraformaldehyde in PBS for 15 minutes, permeablized in 0.2% Triton X-100 in PBS for
2 minutes, washed thrice in PBS for 5 minutes, and mounted in Vectashield mounting
media with DAPI (Vector, Burlingame, CA). Samples were then imaged with an
Olympus FV1000 microscope and three-dimensionally deconvoluted using Autodeblur
software (Media Cybernetics, Bethesda, MD).

**Video microscopy and colony edge imaging**

To examine random cell motility, serial dilutions of *Dictyostelium* cells were
mixed with *K. aerogenes* bacteria in SM broth and grown in 8 well glass slides overnight.
Conditions were selected in which *Dictyostelium* cells were spaced by approximately 40
microns and the ratio of bacteria to *Dictyostelium* was approximately 10:1. Cells were
then imaged using an inverted microscope with a 10x objective and cell movement was
recorded using a Panasonic CCTV VW-BC200 camera. Distances between the
approximate centroids of cells during five minute intervals were measured and scaled to a
stage micrometer to determine random motility values in microns. For colony edge
imaging, 1 μl volumes of serially diluted *Dictyostelium* in HL5 media were allowed to
settle as a small spot in the middle of the well on 8 well glass slides for 15 minutes, then
300 µl of *K. aerogenes* culture in SM media was added to the well, and cells were grown overnight and subsequently imaged with an inverted microscope using a 20x phase contrast objective.

**Statistics**

All statistics were done with Prism (GraphPad Software, San Diego, CA). Unless otherwise noted, one-way ANOVA using Tukey’s test was used to compare differences between multiple groups, and t-tests were used to compare differences between two groups. Significance was defined as p < 0.05.

**Results**

*qkgA*− cells show proliferative phenotypes like *aprA*− and *cfaD*− cells

As the loss of functional components of the AprA/CfaD signal transduction pathways may result in an increased rate of proliferation, we have been examining mutants reported to have a fast-proliferation phenotype to determine if the mutants may have defects in AprA and/or CfaD signaling. Abe et al. disrupted the gene encoding the ROCO protein QkgA to validate a novel disruption strategy (Abe et al., 2003), and the resulting *qkgA*− cells were observed to proliferate rapidly. I also observed that *qkgA*− cells had a significantly faster doubling time than wild-type cells and saw that *qkgA*− cells, like *aprA*− and *cfaD*− cells (Bakthavatsalam et al., 2008; Brock and Gomer, 2005), reached a significantly higher stationary density than wild-type cells in axenic culture (Figure 2 and Table 1). The observed doubling time and stationary density of *qkgA*− cells were not significantly different from *aprA*− cells examined simultaneously (p > 0.05,
Figure 2: QkgA slows proliferation and lowers the stationary density of cells.

Log phase cells were inoculated into HL5 media at $1 \times 10^5$ cells/mL and cell densities were measured daily. Values are mean ± SEM, n ≥ 3 for all conditions. WT indicates wild type.
Table 1. The effect of QkgA on the doubling time and stationary density of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Doubling time, hours</th>
<th>Maximum observed cell density, $10^6$ cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>13.2 ± 0.3</td>
<td>24.5 ± 0.9</td>
</tr>
<tr>
<td>aprA−</td>
<td>10.9 ± 0.1**</td>
<td>44.1 ± 1.7**</td>
</tr>
<tr>
<td>qkgA−</td>
<td>11.5 ± 0.3*</td>
<td>40.1 ± 2.4**</td>
</tr>
<tr>
<td>qkgA− / actin15:QkgA-Gfp</td>
<td>12.7 ± 0.2</td>
<td>33.6 ± 2.2*</td>
</tr>
<tr>
<td>actin15:QkgA-Gfp</td>
<td>16.2 ± 0.9**</td>
<td>15.8 ± 1.2*</td>
</tr>
</tbody>
</table>

Doubling times and stationary densities were calculated as described in the Materials and Methods. Values are mean ± SEM from five or more independent experiments. * indicates that the difference between the value and the wild-type value is significant with p < 0.05, and ** indicates p < 0.01 (one-way ANOVA, Tukey’s test).
one-way ANOVA, Table 1), further suggesting that AprA and QkgA may function in a common proliferation-inhibiting mechanism.

To attempt to rescue the phenotypes of \( qkgA^- \) cells and to determine the phenotype of overexpression of QkgA, I transformed cells with a QkgA-GFP fusion construct (with GFP at the C-terminus of QkgA) under the control of the \( actin15 \) promoter (van Egmond and van Haastert, 2010) and confirmed expression by fluorescence microscopy (Figure 3). The fusion protein showed what appeared to be a cytosolic or vesicular localization (Figure 3B). Expression of QkgA-GFP in wild-type cells resulted in slow proliferation and a low stationary density as compared to wild type (Figure 2 and Table 1). Expression of QkgA-GFP in \( qkgA^- \) cells resulted in a reduced rate of proliferation, with cells exhibiting a log phase doubling time significantly less than that of \( qkgA^- \) cells (\( p < 0.05 \), one-way ANOVA). These results support the hypothesis that QkgA is a negative regulator of proliferation.

\( qkgA^- \) cells exhibit fast proliferation on lawns of bacteria

In addition to fast proliferation in axenic shaking culture, \( aprA^- \) cells proliferate significantly faster than wild-type cells when grown on a lawn of bacteria (Brock and Gomer, 2005), although \( cfaD^- \) cells show no significant difference in proliferation as compared to wild type under these conditions (Bakthavatsalam et al., 2008). To determine whether QkgA affects proliferation of cells grown on bacterial lawns, I spread 1000 cells on agar with bacteria and measured the total number of cells per plate daily. In contrast to previous results, I saw no lag phase during proliferation on bacteria,
Figure 3: Expression of QkgA-GFP in wild-type and qkgA− cells. (A) Cells of the indicated genotype were grown in low-fluorescence axenic media for 24 hours and then washed once in media and imaged by fluorescence microscopy using a 40x objective. Scale bar is 50 μm. (B) qkgA− / act15::QgkA-GFP cells grown in HL5 media were fixed in 4% paraformaldehyde and imaged with a 60x objective. The image was subsequently three-dimensionally deconvoluted. Scale bar is 1 μm.
possibly due to a larger quantity of bacteria spread with the cells. After 24 hours, \( qkgA^- \) cells had proliferated more than wild type, and expression of QkgA-GFP in \( qkgA^- \) cells rescued this increase in proliferation (Figure 4). Expression of QkgA-GFP in wild-type cells did not significantly slow proliferation on bacteria. The proliferation between genotypes during subsequent 24-hour periods was not significantly different. These results indicate that the absence of QkgA results in fast proliferation on bacteria when cell numbers are low.

\( qkgA^- \) cells secrete AprA and CfaD

One potential explanation for a fast proliferation phenotype is a decrease in the extracellular accumulation of AprA or CfaD relative to wild type. To determine if low extracellular levels of AprA or CfaD might be responsible for the fast proliferation of \( qkgA^- \) cells, I examined extracellular levels of AprA and CfaD in \( qkgA^- \) cells. Both AprA and CfaD accumulate in the medium of \( qkgA^- \) cells at levels comparable to wild type levels (Figure 5), strongly suggesting that the rapid proliferation of \( qkgA^- \) cells is not due to altered levels of extracellular AprA or CfaD.

\( qkgA^- \) cells are insensitive to proliferation inhibition by AprA and CfaD

If AprA and/or CfaD signal through QkgA to repress proliferation, it is expected that \( qkgA^- \) cells would show some degree of insensitivity to AprA and/or CfaD. To test this prediction, I incubated proliferating cells with recombinant AprA (rAprA) or rCfaD and determined the percent decrease in cell density as compared to a buffer control after a 16-hour incubation. Wild-type cells exhibited an approximately 15 percent decrease in
Figure 4: QkgA slows proliferation on bacterial lawns. 1000 cells were plated on SM/5 plates with \textit{K. aerogenes} bacteria and the total number of cells was determined at 24 hours. * indicates a significance of $p < 0.05$, and ** indicates a significance of $p < 0.01$ (repeated measures one-way ANOVA, Tukey’s test).
**Figure 5: qkgA− cells secrete AprA and CfaD.** Conditioned media from wild-type and qkgA− cells at (A) log phase (3 x 10⁶ cells/mL) or (B) post-log phase (12 x 10⁶ cells/mL) was assayed by Western blots with anti-AprA antibodies (left) or anti-CfaD antibodies (right). Data are representative of three independent experiments. Asterisk indicates a breakdown product of CfaD. Markers at left indicate the position of molecular mass markers in kDa.
cell density in response to rAprA or rCfaD (Figure 6), which is on the order of what we have previously observed (Bakthavatsalam et al., 2008; Bakthavatsalam et al., 2009; Choe et al., 2009). However, no inhibition of \( qkgA^- \) cell proliferation by either rAprA or rCfaD was detected. Although rAprA appeared to slightly increase the proliferation of \( qkgA^- \) cells, paired t-tests of cell densities after adding either rAprA/rCfaD or buffer showed no significant differences in cell density between the two conditions for \( qkgA^- \) cells, indicating that rAprA and rCfaD have no significant effect on the proliferation of \( qkgA^- \) cells. Expression of QkgA-GFP in \( qkgA^- \) cells restored the ability of rAprA or rCfaD to inhibit proliferation, strongly suggesting that the insensitivity of \( qkgA^- \) cells to rAprA and rCfaD is due specifically to a nonfunctional \( qkgA \) gene. These results support the hypothesis that QkgA is required for AprA and CfaD-mediated inhibition of proliferation.

\( qkgA^- \) cells are multinucleate

\( aprA^- \) and \( cfaD^- \) cells exhibit a multinucleate phenotype (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). To determine whether \( qkgA^- \) cells show this phenotype, I counted the number of nuclei per cell of DAPI-stained log phase cells. \( qkgA^- \) cells were significantly less likely to have one nucleus per cell than wild type, and significantly more likely to have either two nuclei or three or more nuclei per cell than wild type (Table 2). Expression of QkgA-GFP in \( qkgA^- \) cells rescued this multinucleate phenotype. Overexpression of QkgA-GFP in wild-type cells resulted in significantly fewer binucleate cells as compared to wild type. These results indicate that QkgA negatively regulates the number of nuclei per cell, perhaps by inhibiting mitosis.
Figure 6: Expression of QkgA-GFP rescues the insensitivity of qkgA− cells to rAprA (left) and rCfaD (right). Proliferating cells were incubated for 16 hours with either 1000ng/mL rAprA or 600ng/mL rCfaD or an equivalent volume of buffer, and cell densities were then determined. The percent inhibition of cell density as compared to a buffer control is shown. Values are mean ± SEM, n ≥ 4 for all conditions. * indicates a significance of p < 0.05, ** indicates a significance of p < 0.01 and *** indicates significance of p < 0.001 (one-way ANOVA, Tukey’s test).
Table 2. The effect of QkgA on the number of nuclei per cell.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>1</th>
<th>2</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>78 ± 2</td>
<td>20 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>qkgA</td>
<td>60 ± 2***</td>
<td>30 ± 1**</td>
<td>9 ± 1**</td>
</tr>
<tr>
<td>qkgA^- / actin15:QkgA-Gfp</td>
<td>81 ± 2</td>
<td>18 ± 2</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>actin15:QkgA-Gfp</td>
<td>85 ± 1</td>
<td>14 ± 1*</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

Log phase cells were fixed and stained with DAPI, and the number of nuclei per cell of at least 200 cells per genotype was determined by fluorescence microscopy. Values are the mean ± SEM from three independent experiments. * indicates that the difference between the value and the wild-type value is significant with p<0.05, ** indicates p <0.01, and *** indicates p<0.001 (one-way ANOVA, Tukey’s test).
Additionally, these results show further phenotypic similarities between \( qkgA^- \) cells and \( aprA^- \) and \( cfaD^- \) cells, supporting the hypothesis that QkgA mediates at least some aspects of AprA and CfaD-induced signaling.

**QkgA does not negatively regulate cell growth on a per nucleus basis**

Cell growth, defined as the accumulation of mass or protein, and cell proliferation, the increase in cell number, are often interdependent properties but can be regulated independently (Jorgensen and Tyers, 2004). Although \( aprA^- \) and \( cfaD^- \) cells are more massive than wild-type cells, mass and protein are not accumulated in these genotypes at a rate faster than wild type on a per nucleus basis, suggesting that AprA and CfaD do not regulate growth (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). To determine the effect of QkgA on the mass and protein content of cells, I examined these properties in log phase cells. Protein content of wild-type cells was identical to that previously observed (Bakthavatsalam et al., 2008; Bakthavatsalam et al., 2009), although I observed lower cell mass values than were previously seen, which may have been due to mass measurement in our study using cells in HL5 medium as opposed to cells washed in phosphate buffer or water. Like \( aprA^- \) and \( cfaD^- \) cells, \( qkgA^- \) cells were more massive than wild type, though differences in protein content between genotypes were not significant (Table 3). Expression of QkgA-GFP in \( qkgA^- \) cells resulted in cells with roughly wild-type levels of cell mass, and overexpression of QkgA-GFP in the wild-type background had no significant effect on cell mass or protein content. On a per nucleus basis, no significant differences were observed between wild-type and \( qkgA^- \) cells in mass or protein content.
Table 3. The effect of QkgA on the mass and protein content of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Per 10^7 cells</th>
<th>Per 10^7 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td>Protein (mg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>5.18 ± 0.24</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>qkgA</td>
<td>7.15 ± 0.16**</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>qkgA^- / actin15:QkgA-Gfp</td>
<td>5.54 ± 0.64</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>actin15:QkgA-Gfp</td>
<td>4.83 ± 0.62</td>
<td>0.27 ± 0.02</td>
</tr>
</tbody>
</table>

Mass and protein content were determined as described in the Materials and Methods. The number of nuclei per 100 cells was calculated from the data presented in Table 2. Values are the mean ± SEM from three or more independent experiments. * indicates that the difference between the value and the wild-type value is significant with p<0.05 and ** indicates p <0.01 (one-way ANOVA, Tukey’s test).
To examine the effect of QkgA on cell growth, I assumed a steady state average cell size (i.e., that a doubling in cell number results in a doubling of mass, protein, or nuclei) and divided the mass, protein, or nuclei content of cells of different genotypes by the calculated log phase doubling times to estimate the mass, protein, or nuclei increase per hour. On a per cell basis, qkgA− cells showed a significantly higher mass accumulation than wild type, while expression of QkgA-GFP in qkgA− cells caused a mass accumulation like wild type (Table 4). qkgA− cells accumulated nuclei more rapidly than wild-type cells, and overexpression of QkgA-GFP in the wild-type background resulted in a rate of nuclear accumulation lower than wild type. When examined on a per nucleus basis, the mass and protein accumulation per hour of wild-type and qkgA− cells were not significantly different (p > 0.05, one-way ANOVA). These results suggest that, like AprA and CfaD, QkgA negatively regulates proliferation but does not significantly affect cell growth on a per nucleus basis.

QkgA does not affect spore viability

Following development, aprA− and cfaD− cells yield fewer detergent-resistant spores than wild type (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). I examined the ability of qkgA− cells to generate detergent-resistant spores by allowing an equivalent number of wild-type and qkgA− cells to develop, collecting spores, and plating dilutions of detergent-treated spores. The number of visible phase-dark, ovoid spores collected following development was 102 ± 4 percent of the input cell number for wild type and 117 ± 1 percent for qkgA− cells (mean ± SEM, n = 3). Although the difference between the number of recovered spores from wild type and qkgA− cells was significant (p < 0.05,
Table 4. The effect of QkgA on the mass and protein accumulation of cells.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Per 10^7 cells per hour</th>
<th>Per 10^7 nuclei per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td>Protein (µg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.39 ± 0.02</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>qkgA^-</td>
<td>0.62 ± 0.02**</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>qkgA^-/actin15:QkgA-Gfp</td>
<td>0.44 ± 0.05</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>actin15:QkgA-Gfp</td>
<td>0.30 ± 0.04</td>
<td>17 ± 1</td>
</tr>
</tbody>
</table>

Mass, protein, and nuclei values from Table 3 were divided by the observed doubling time of the respective genotype. Doubling times were calculated as described in Materials and Methods. Values are the mean ± SEM from three or more independent experiments. * indicates that the difference between the value and the wild-type value is significant with p<0.05 and ** indicates p <0.01 (one-way ANOVA, Tukey’s test).
t-test), this significance was lost when the values were considered on a per nucleus basis by dividing the percentage of recovered spores to input cells by the observed number of nuclei per cell for each genotype (as calculated from Table 2), suggesting that this difference was the result of the multinucularity of \( qkgA^- \) cells. As judged by plaque number after plating on bacteria, the number of viable spores after detergent treatment was 67 ± 5 percent that of the number of spores plated for wild-type and 68 ± 9 percent for \( qkgA^- \) cells (mean ± SEM, n = 3). Differences in detergent-resistant spore number between wild-type and \( qkgA^- \) cells were not significant (t-test). These results suggest that QkgA does not affect the development of spores.

**Colonies of cells lacking AprA, CfaD, or QkgA expand slowly**

I noticed that, despite a rapid proliferation phenotype, \( aprA^- \) and \( cfaD^- \) cells appeared to expand as a colony on a lawn of bacteria less rapidly than wild type. To examine this apparent phenotype, and to determine if \( qkgA^- \) cells are similar, I plated serial dilutions of cells on bacterial lawns and measured the size of colonies. Under these conditions, the expansion of colonies of \( aprA^- \) and \( cfaD^- \) cells was less than that of wild type (Figure 7). Additionally, colonies of \( qkgA^- \) cells showed a rate of expansion less than wild type, but similar to that of \( cfaD^- \) and \( aprA^- \) cells. These results suggest that, although AprA, CfaD, and QkgA inhibit proliferation, a drawback to not expressing these proteins is a decreased ability of colonies of cells to expand under at least some conditions.
Figure 7: AprA, CfaD, and QkgA affect the expansion of colonies on bacterial lawns. Serial dilutions of log phase cells in shaking culture (1-4 x 10^6 cells/mL) were mixed with bacteria and spread on SM/5 plates, and the average diameter of well-spaced colonies was determined daily. Values are mean ± SEM, n ≥ 3. The absence of error bars indicates that the error was smaller than the plot symbol. For wild type, plates were overgrown after day 8 and colony boundaries could not be determined after this point.
As a reduced colony expansion phenotype could be due to defects in cell motility, I examined random motility of cells cultured at low densities with bacteria on glass slides using video microscopy. During a five minute interval, I observed an average displacement of wild-type, $aprA^-$, $cfaD^-$, and $qkgA^-$ cells of $7.6 \pm 0.6 \ \mu m$, $10.4 \pm 1.1 \ \mu m$, $8.6 \pm 0.6 \ \mu m$, and $10.5 \pm 0.7 \ \mu m$, respectively (means $\pm$ SEM from three independent experiments with at least ten cells per condition). Differences in observed motilities were not significant between any of the genotypes tested ($p > 0.05$, Tukey’s test). These results show that $aprA^-$, $cfaD^-$, and $qkgA^-$ cells exhibit random motility comparable to wild type and suggest that the reduced colony expansion observed in these mutants is not due to a defect in cell motility.

I used a similar experimental setup to examine the morphology of Dictyostelium colonies at the interface of populations of Dictyostelium and bacteria. At the edges of wild type colonies, some of the Dictyostelium cells were dispersed into the bacterial lawn, and the boundary between Dictyostelium cells and bacteria was poorly defined (Figure 8). In contrast, boundaries between bacteria and $aprA^-$, $cfaD^-$, or $qkgA^-$ cells were well defined and invasion by Dictyostelium cells into the bacteria was not evident. Together, these results indicate that AprA, CfaD, and QkgA affect the expansion of cell colonies but not the random motility of individual cells.

Discussion

Like $aprA^-$ and $cfaD^-$ cells, $qkgA^-$ cells have a rapid proliferation phenotype (Abe et al., 2003). I found that $qkgA^-$ cells, like $aprA^-$ and $cfaD^-$ cells, also have as a
Figure 8: AprA, CfaD, and QkgA affect cell dispersal at the borders of colonies during growth with bacteria. Small spots of wild-type, aprA<sup>−</sup>, cfaD<sup>−</sup>, or qkgA<sup>−</sup> cells were grown overnight in glass culture chambers with <i>K. aerogenes</i> bacteria, and interfaces between bacteria and <i>Dictyostelium</i> cell populations were imaged. Scale bar is 100 microns.
high stationary density in shaking culture, multinuclearity and rapid accumulation of nuclei, a rate of growth per nucleus like that of wild type, and a reduced rate of colony expansion when grown with bacteria. In addition, QkgA is required for the proliferation-inhibiting activity of AprA and CfaD. Together, these results suggest that QkgA may serve as a signal-transducing component downstream of AprA and CfaD.

$qkgA^-$ cells proliferate more rapidly than wild type cells and have a higher stationary phase density, whereas wild type cells overexpressing QkgA-GFP have a slower proliferation and a lower stationary phase cell density. These results suggest that the effects of QkgA on proliferation and stationary density are sensitive to the dosage of QkgA. I saw that $qkgA^-$ cells expressing QkgA-GFP proliferate like wild type cells during log phase growth, although $qkgA^-/ QkgA$-GFP cells proliferate to a higher stationary density than wild type. It may be the case, therefore, that native QkgA has increased activity at higher cell densities, whereas the ectopically expressed QkgA-GFP fusion protein does not. Alternatively, QkgA-GFP may be only partially active or not targeted correctly, or, as these experiments were done in the absence of selective drugs, QkgA-GFP expression may be reduced at high cell densities due to plasmid loss.

Like $aprA^-$ and $cfaD^-$ cells, $qkgA^-$ cells tend to be multinucleate. Although a cytokinesis defect could be responsible for this phenotype, this seems unlikely, as cells with a cytokinesis defect would be expected to proliferate slowly rather than rapidly. I observed that QkgA inhibits the accumulation of nuclei per cell. If cells cannot complete
cytokinesis as fast as they produce new nuclei, this could lead to an increased number of nuclei per cell, which could explain the multinucleate phenotype of \( qkgA^- \) cells.

When estimating the accumulation of mass and protein per hour on a per nucleus basis, I saw that \( qkgA^- \) cells showed a higher mass and protein accumulation than wild type, though these differences were not significant, suggesting that \( qkgA^- \) cells accumulate mass and protein per nucleus at a rate like wild type. The mass of wild-type cells increases as a function of cell density (Soll et al., 1976). As \( qkgA^- \) cells proliferate rapidly but accumulate mass at a rate like wild type, QkgA may thus affect the ability of cells to increase in mass as a function of density. It is unclear whether this increase in mass provides an advantage to wild-type cells, as our data shows that \( qkgA^- \) cells at stationary density do not die more rapidly than wild type and that \( qkgA^- \) cells do not show a defect in generating viable spores.

As \( aprA^- \), \( cfaD^- \), and \( qkgA^- \) cells proliferate more rapidly than wild-type cells, AprA, CfaD, and QkgA almost certainly serve some fitness-increasing function, or else cells with these functional proteins would likely be selected against. Whereas \( aprA^- \) and \( cfaD^- \) cells yield less viable spores after development than wild type, \( qkgA^- \) cells do not show a defect in spore viability, suggesting that QkgA has not been selected for due to a role in the generation of viable spores. However, I saw that colonies of \( aprA^- \), \( cfaD^- \), and \( qkgA^- \) cells showed a reduced rate of expansion when grown on bacterial lawns. The ability of a population of cells to expand rapidly almost certainly provides an advantage in an environment with low nutrient concentrations, as a population of cells must require
a certain quantity of nutrients in order to continue increasing in number at an optimal rate, and the cell population depends upon expansion to attain this quantity of nutrients. Thus, although \textit{aprA}^-, \textit{cfaD}^-, and \textit{qkgA}^- cells may proliferate more rapidly than wild type, these cells could potentially be at a disadvantage due to their reduced ability to expand as a colony. The mechanism by which these genes function in colony expansion is unclear. As I saw that \textit{qkgA}^- cells proliferate more than wild type after a 24-hour period of growth in the presence of bacteria, it seems unlikely that \textit{qkgA}^- cells are deficient in utilizing bacteria as an energy source. \textit{aprA}^-, \textit{cfaD}^-, and \textit{qkgA}^- cells aggregate to form fruiting bodies and show random cell motility in the presence of bacteria similar to wild-type cells, indicating that these genes are not essential for cell motility. It may be the case that the loss of these genes affects the ability of cells to chemotax toward bacteria, or that AprA and/or CfaD function as chemorepellants and thus aid in the dispersal of a population of cells. Alternatively, rapid proliferation in itself may reduce colony expansion by reducing the local nutrient concentration available to cells, causing cell starvation and/or precocious development. Interestingly, cells lacking the heterotrimeric G protein complex subunit G β, which we have shown to be essential for AprA signaling (Bakthavatsalam et al., 2009), or the kinase YakA also proliferate rapidly in culture (Bakthavatsalam et al., 2009; Souza et al., 1998) but show a reduced colony expansion phenotype (van Es et al., 2001; Wu et al., 1995).

Currently, we cannot distinguish whether QkgA is actively involved in AprA/CfaD signaling (for instance, in a phospho-cascade) or whether QkgA plays a permissive role, such as the establishment or maintenance of some component of
AprA/CfaD signaling. However, the fact that \(qkgA^-\) cells proliferate rapidly suggests that QkgA functions specifically in negative regulation of proliferation as opposed to a general function of cell metabolism or maintenance that affects multiple cellular processes, as one might predict that the loss of function of such a gene would have pleiotropic effects and thus lead to unfit or inviable cells. Therefore, it seems likely that QkgA functions specifically to slow proliferation and therefore may be an integral part of the AprA/CfaD-mediated mechanism of proliferation inhibition.

Together, our results indicate that the ROCO family kinase QkgA negatively regulates both proliferation and stationary density and is essential for the function of the proliferation-inhibiting autocrine signals AprA and CfaD. To our knowledge, no ROCO family kinase other than QkgA has been rigorously characterized as a regulator of proliferation. However, an RNA interference-mediated screen of the \(Drosophila melanogaster\) kinome for genes that mediate cell cycle progression identified a ROCO kinase family member as a potential regulator of cell cycle phase and centrosomal function (Bettencourt-Dias et al., 2004). It therefore may be the case that regulation of proliferation by ROCO kinases is conserved in metazoans and that other ROCO proteins may have functions analogous to those of QkgA in \(Dictyostelium\).
Chapter 3. The putative transcription factor BzpN slows proliferation and functions in the regulation of cell density by AprA and CfaD¹

Introduction

Basic leucine zipper (bZIP) transcription factors are a large family of proteins that function in a wide range of signal transduction pathways (Jakoby et al., 2002; Kerr et al., 1992) and are defined by an approximately 30 amino-acid sequence with a leucine residue at every seventh position, which mediates homo- or heterodimerization through alpha-helical interactions (Landschulz et al., 1988) and a stretch of adjacent basic residues that mediate DNA binding (Busch and Sassone-Corsi, 1990). The *Dictyostelium* genome encodes 19 predicted bZIP transcription factors. Little is known regarding the function of the majority of these bZIP proteins, though the bZIP proteins DimA and DimB are downstream effectors of the prestalk cell fate-specifying signal DIF and translocate to the nucleus in response to this signal (Huang et al., 2006; Thompson et al., 2004; Zhukovskaya et al., 2006). Our colleague Gadi Shaulsky found that cells lacking BzpN proliferate rapidly, and I investigated whether BzpN is involved in AprA/CfaD signaling. I found that BzpN plays a role in AprA- and CfaD-mediated inhibition of proliferation. *bzpN*⁻ cells proliferate rapidly, show aberrant responses to rAprA and rCfaD, and are multinucleate. However, *bzpN*⁻ cells do not show defects in spore viability and do not proliferate to a higher stationary density than wild type. Thus BzpN mediates a subset of the effects of AprA and CfaD.

¹ The work in this chapter has been published (Phillips et al., 2011).
Materials and Methods

To measure proliferation in response to stationary phase conditioned media, wild-type cells were grown to a density of 20 - 25 x 10^6 cells/mL in HL5. Cells were removed by centrifugation at 800 x g for 3 minutes at room temperature and the conditioned medium supernatant was collected and sterilized by passage through a 0.2 micron filter (Pall, Ann Arbor, MI). Wild-type, \( bzpN^- \), or \( bzpN^- / bzpN-GFP \) cells were then resuspended in this conditioned media in 5 mL volumes at 0.5 x 10^6 cells/mL, and cell density was determined by hemocytometer after a 24 hour incubation in shaking culture at 22° C.

Fluorescence microscopy

To determine the effect of conditioned media on BzpN-GFP localization, conditioned media from wild-type, \( aprA^- \), and \( cfaD^- \) cells at a density of 20 - 25 x 10^6 cells/mL was collected as described above. \( bzpN^- / act15::bzpN-GFP \) cells at a density of 0.5 - 4 x 10^6 cells/mL were resuspended in 0.5 ml volumes at 0.5 x 10^6 cells/mL in the media in which they had been growing, in fresh HL5, or in conditioned media from wild-type, \( aprA^- \), or \( cfaD^- \) cells. These cultures were incubated overnight at 22° C in shaking culture, and then 200 μL of each culture was allowed to settle in one well of an 8-well chamber slide (Nunc) for 30 minutes. The medium was removed and cells were then fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 minutes, permeabilized with 0.1% NP-40 in PBS pH 7.4 for 2 minutes, and washed three times in PBS pH 7.4. Cells were then mounted in Vectashield mounting media with DAPI (Vector, Burlingame, CA) and imaged with an Olympus FV1000 fluorescence microscope. To examine BzpN-GFP
localization in comparison to lysosomal or mitochondrial localization, $bzpN^{-}/bzpN\text{-GFP}$ cells at $0.5 - 4 \times 10^6$ cells/mL in shaking culture were resuspended in low-fluorescence media (Bokko et al., 2007) at $0.5 \times 10^6$ cells/mL with either 500 nM Lysotracker Red dye (Invitrogen) or 500 nM Mitotracker Red dye (Invitrogen) and incubated in shaking culture at $22^\circ C$ for 2 hours. Cells were then washed once in low-fluorescence media and then fixed and imaged as described above.

**Results**

**BzpN regulates proliferation**

To gain insight into the function of basic leucine zipper transcription factors encoded in the *Dictyostelium* genome, genes encoding bZIP proteins were targeted for disruption by homologous recombination (Huang et al., 2006). The $BzpN$ gene encodes a 999-amino acid protein that has 33% identity and 56% similarity over a 59-amino acid segment to the human bZIP protein Nrf2, which mediates cellular responses to oxidative stress (Sykiotis and Bohmann, 2010). A $BzpN$ null mutant was generated by our colleague Gadi Shaulsky and confirmed by diagnostic PCR and RT-PCR (Phillips et al., 2011). $bzpN^{-}$ cells exhibited rapid proliferation as compared to wild-type cells. To determine if the rapid proliferation was due to loss of BzpN, I constructed an extrachromosomal vector in which the constitutive *actin15* promoter drives expression of BzpN with GFP fused to the C-terminus, transformed wild-type and $bzpN^{-}$ cells with this vector, and examined the proliferation of these cell lines. In shaking culture, $bzpN^{-}$ cells showed faster proliferation than wild-type cells during the logarithmic phase of proliferation and had a significantly faster doubling time (Figure 9 and Table 5).
**Figure 9: BzpN slows the proliferation of cells.** Log phase cells were diluted to $1 \times 10^5$ cells/mL and grown in shaking culture. Values are means ± SEM ($n \geq 4$) for all conditions. WT indicates wild type. All strains are in the AX4 background.
Table 5. The effect of BzpN on the doubling time and stationary density of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Doubling time, hours</th>
<th>Maximum observed cell density, $10^6$ cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>12.1 ± 0.4</td>
<td>22.6 ± 1.8</td>
</tr>
<tr>
<td>$bzpN^{-}$</td>
<td>10.5 ± 0.2*</td>
<td>25.2 ± 1.3</td>
</tr>
<tr>
<td>$bzpN^{-} / actin15::bzpN-GFP$</td>
<td>13.7 ± 0.9</td>
<td>25.0 ± 2.5</td>
</tr>
<tr>
<td>$actin15::bzpN$-GFP</td>
<td>14.0 ± 0.4</td>
<td>14.4 ± 0.6*</td>
</tr>
</tbody>
</table>

Doubling times and stationary densities were calculated for the proliferation curves in Figure 9. Values are mean ± SEM from four or more independent experiments. * indicates that the difference between the value and the wild-type value is significant with $p < 0.05$ (one-way ANOVA, Tukey’s test).
However, \textit{bzpN}− cells did not proliferate to a higher cell density than wild type (Table 5), in contrast with \textit{aprA}− and \textit{cfaD}− cells (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). The expression of BzpN-GFP in \textit{bzpN}− cells resulted in a proliferative phenotype and doubling time similar to wild-type cells, strongly suggesting that the rapid proliferation phenotype of \textit{bzpN}− cells is due specifically to the absence of BzpN. Although \textit{bzpN}− / \textit{act15::bzpN-GFP} cells did not show a reduced maximum cell density as compared to wild type, expression of BzpN-GFP in wild-type cells resulted in a significantly reduced maximum cell density (Table 5, Figure 9), suggesting that dosage of BzpN affects proliferation. \textit{bzpN}− cells also proliferated faster than wild-type cells when grown on bacterial lawns, and this rapid proliferation was rescued by the expression of BzpN-GFP (Figure 10). Together, these results indicate that BzpN functions to slow the proliferation of cells during exponential growth.

\textit{bzpN}− cells do not show a deficiency in AprA or CfaD accumulation

One explanation for the rapid proliferation of \textit{bzpN}− cells could be reduced extracellular AprA and/or CfaD accumulation due to aberrant expression, secretion, or degradation of these proteins. However, wild-type and \textit{bzpN}− cells showed similar levels of extracellular AprA and CfaD (Figure 11). A 27 kD breakdown product of CfaD (Bakthavatsalam et al., 2008) appeared at higher levels in both \textit{bzpN}− and \textit{bzpN}− / \textit{act15::bzpN-GFP} conditioned media as compared to wild-type, although this difference likely does not significantly affect proliferation, as suggested by the similar proliferation rates of wild-type and \textit{bzpN}− / \textit{act15::bzpN-GFP} cells. Silver staining of conditioned media samples showed similar protein levels, indicating equal loading and showing
Figure 10: BzpN slows proliferation on bacterial lawns. 1000 cells were spread on agar plates with bacteria. After 24 hours, the *Dictyostelium* cells were collected and counted. Values are means ± SEM (n ≥ 3). The differences in cell numbers between wild-type and *bzpN*− and between *bzpN*− and *bzpN*−/*bzpN*-GFP are significant (p < 0.05, one-way ANOVA, Tukey’s test).
Figure 11: \textit{bzpN}⁻ cells secrete Ap\textit{r}A and C\textit{fa}D. Cells were grown to $12 \pm 1 \times 10^6$ cells/mL in shaking culture and conditioned medium was collected and assayed by Western blot with anti-Ap\textit{r}A or anti-C\textit{fa}D antibodies. Asterisk indicates a 27 kDa breakdown product of C\textit{fa}D. The intensity of this 27 kDa band and the light band at 50 kDa were variable among experiment replicates. Silver staining of conditioned media samples was done as a loading control. Numbers at the left indicate molecular mass in kDa. Data are representative of three independent experiments.
wild-type levels of extracellular protein accumulation in \(bzpN^-\) cells. These results suggest that BzpN is not essential for normal extracellular accumulation of AprA and CfaD, and that the fast proliferation of \(bzpN^-\) cells is not due to a lack of extracellular AprA or CfaD.

\(bzpN^-\) cells show aberrant responses to AprA and CfaD

If BzpN is a component of an AprA/CfaD signal transduction pathway, \(bzpN^-\) cells could be insensitive to the proliferation-inhibiting effects of AprA or CfaD. To test this possibility, I incubated wild-type, \(bzpN^-\), or \(bzpN^-/act15::bzpN-GFP\) cells at low density with rAprA or rCfaD and determined the ability of these proteins to inhibit proliferation. As previously observed, rAprA and rCfaD inhibited the proliferation of wild-type cells (Bakthavatsalam et al., 2009; Phillips and Gomer, 2010) (Figure 12). However, rAprA had no significant effect on the proliferation of \(bzpN^-\) cells, whereas rCfaD significantly increased \(bzpN^-\) cell proliferation. In contrast, both rAprA and rCfaD inhibited the proliferation of \(bzpN^-/act15::bzpN-GFP\) cells at levels similar to wild-type. These results indicate that BzpN is necessary for the proliferation-inhibiting activity of AprA and CfaD and suggest that BzpN may function downstream of AprA and CfaD in a signal transduction pathway.

\(bzpN^-\) cells are multinucleate and accumulate mass at a rate like wild type

Both \(aprA^-\) and \(cfaD^-\) cells have more nuclei per cell than wild type, which may be caused by rapid mitosis without a concomitant increase in the rate of cytokinesis (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). To determine whether \(bzpN^-\)
Figure 12: *bzpN*<sup>−</sup> cells show aberrant responses to AprA and CfaD. Cells were incubated with either rAprA, rCfaD, or an equivalent volume of buffer. Cell densities were measured after 48 hours, and the percent inhibition of proliferation by AprA or CfaD as measured against a buffer control was calculated. Values are mean ± SEM (n ≥ 4). For both AprA and CfaD, differences between wild type and *bzpN*<sup>−</sup> cells are significant (p<0.05, 1-way ANOVA, Tukey’s test), whereas differences between wild type and rescue are not. For *bzpN*<sup>−</sup> cells, rCfaD but not rAprA significantly increased proliferation (p < 0.05, paired t-test).
cells share this phenotype, I examined the number of nuclei per cell for wild type, $bzpN^-$ cells, and $bzpN^- / act15::bzpN-GFP$ cells. $bzpN^-$ cells had significantly more nuclei per cell than wild type, whereas $bzpN^- / act15::bzpN-GFP$ cells showed no significant difference in nuclei per cell compared to wild type (Table 6). These results indicate that, like AprA and CfaD, BzpN reduces the average number of nuclei per cell.

Although growth (the accumulation of mass) is essential for continued proliferation, cell growth and proliferation can be regulated independently (Su and O'Farrell, 1998). $aprA^-$ and $cfaD^-$ cells show an accumulation of mass per nucleus like that of wild type, suggesting that AprA and CfaD regulate proliferation but not growth (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). To determine the effect of BzpN on growth, I measured the mass and protein content of log phase cells and calculated the accumulation of mass and protein per cell and per nucleus using the measured doubling times (Table 5) and nuclei counts (Table 6). AX4, the parental strain of $bzpN^-$, was more massive and had more protein on a per cell basis than we have previously observed for AX2, the parental strain of our previously characterized mutants (Phillips and Gomer, 2010). AX4 also had more nuclei per cell than AX2 (Brock and Gomer, 2005), though mass and protein levels on a per nucleus basis for AX4 are similar to what we have observed for AX2 (Phillips and Gomer, 2010). $bzpN^-$ cells were significantly more massive and had a higher protein content than wild-type or $bzpN^- / act15::bzpN-GFP$ cells (Table 6), though these differences were lost when mass and protein content were considered on a per nucleus basis. I then examined mass and protein accumulation as a function of time by dividing the observed mass and protein measurements by the
Table 6. The effect of BzpN on the mass, protein, and nuclei content of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Per $10^7$ cells</th>
<th>% cells with $n$ nuclei</th>
<th>Nuclei/100 cells</th>
<th>Per $10^7$ nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td>Protein (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>9.0 ± 0.3</td>
<td>0.51 ± 0.02</td>
<td>44 ± 9</td>
<td>168 ± 9</td>
</tr>
<tr>
<td>$bzpN^{-}$</td>
<td>10.7 ± 0.1*</td>
<td>0.63 ± 0.02*</td>
<td>27 ± 4*</td>
<td>199 ± 10*</td>
</tr>
<tr>
<td>$bzpN^{-}/actin15::bzpN-GFP$</td>
<td>9.0 ± 0.2</td>
<td>0.49 ± 0.01</td>
<td>50 ± 5</td>
<td>168 ± 6</td>
</tr>
</tbody>
</table>

Mass and protein content were determined as described in the Materials and Methods. Values are the mean ± SEM from three or more independent experiments. * indicates that the difference between the value and the wild-type value is significant with p<0.05 (one-way ANOVA, Tukey’s test).
measured doubling times, yielding the accumulation of mass or protein per hour. Mass and protein accumulation per cell was greater in AX4 than what we have seen in AX2, though mass and protein accumulation per nucleus for AX4 are similar to what we have seen for AX2 (Phillips and Gomer, 2010). \(bzpN^{-}\) cells accumulated mass, protein, and nuclei more rapidly than wild-type or \(bzpN^{-}/act15::bzpN-GFP\) cells (Table 7). However, differences in the accumulation of mass or protein on a per nucleus basis were not significant between wild-type and \(bzpN^{-}\) cells (Table 7). These results suggest that BzpN, like AprA and CfaD, negatively regulates proliferation but does not significantly regulate growth on a per nucleus basis.

**AprA, CfaD, and QkgA are necessary for proliferation inhibition by BzpN-GFP overexpression**

Overexpression of BzpN in the wild-type background results in slow proliferation and a low stationary phase density (Figure 9). To test whether AprA, CfaD, or QkgA are necessary for proliferation inhibition by BzpN, I expressed BzpN-GFP in cells lacking these proteins, confirmed expression by fluorescence microscopy, and examined the proliferation of these strains in parallel with the parental strains. As our \(aprA^{-}\), \(cfaD^{-}\), and \(qkgA^{-}\) cells are in the AX2 genetic background, I expressed BzpN-GFP in AX2 cells as a control, and saw a similar reduction in maximum cell density as in Figure 9, where the AX4 strain was used (Figure 13). Whereas expression of BzpN-GFP in wild-type cells resulted in significantly lower cell densities during proliferation as compared to untransformed wild-type cells, expression of BzpN-GFP in \(aprA^{-}\), \(cfaD^{-}\), or \(qkgA^{-}\) cells resulted in no significant decrease in cell density during proliferation as compared to
Table 7. The effect of BzpN on the mass and protein accumulation of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Per $10^7$ cells per hour</th>
<th>Per $10^7$ nuclei per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td>Protein (µg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.74 ± 0.03</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>$bzpN^{-}$</td>
<td>1.02 ± 0.02*</td>
<td>59 ± 2*</td>
</tr>
<tr>
<td>$bzpN^{-} / actin15::bzpN-GFP$</td>
<td>0.66 ± 0.04</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

Mass and protein values from Table 2 were divided by the observed doubling time of the respective genotype. Doubling times were calculated as described in Materials and Methods. Values are the mean ± SEM from three or more independent experiments. * indicates that the difference between the value and the wild-type value is significant with $p < 0.05$ (one-way ANOVA, Tukey’s test).
Figure 13: AprA, CfaD, and QkgA are required for inhibition of proliferation by BzpN. Proliferation curves were done as described in Figure 1. Values are mean ± SEM, n ≥ 3. Expression of BzpN-GFP in wild-type cells resulted in a significant decrease in cell density on days 5 through 8 with p < 0.05, whereas expression of BzpN-GFP in aprA−, cfaD−, or qkgA− cells did not cause a significant reduction in cell density at any measured timepoint (t-test). WT indicates wild type. All strains are in the AX2 background. Data for strains expressing BzpN-GFP are the average of two independent transformant clones.
untransformed parental cells (Figure 13). These results suggest that AprA, CfaD, and QkgA are essential for the proliferation-inhibiting activity of BzpN.

**BzpN does not affect the production of spores or the ability of cell colonies to expand**

$aprA^-$ and $cfaD^-$ cells yield fewer viable, detergent-resistant spores than wild type following multicellular development, indicating that AprA and CfaD promote the development of viable spores (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). I tested the effect of BzpN on spore development by allowing wild-type, $bzpN^-$, and $bzpN^- / act15::bzpN-GFP$ cells to develop, collecting and counting spores, and plating detergent-treated spores on lawns of bacteria. For all genotypes, the number of spores collected was higher than the number of cells allowed to develop (Table 8). This is a higher spore yield than we have previously observed, and may be due to the high percentage of multinuclear cells in the Ax4 background, or a round of cell proliferation during development, as has been previously observed (Chen et al., 2004). Differences between any two genotypes for either the number of spores collected or the number of viable detergent-treated spores were not significant (Table 8). These results suggest that BzpN does not significantly affect spore viability and that AprA and CfaD affect spore development by a mechanism independent of BzpN.

Despite the fact that $aprA^-$ and $cfaD^-$ cells proliferate rapidly, colonies of these cells on a lawn of bacteria expand less rapidly than wild-type colonies (Phillips and Gomer, 2010), indicating that AprA and CfaD may function to facilitate the dispersal of groups of cells. To determine whether $bzpN^-$ cells expand slowly as colonies on a lawn
Table 8. The effect of BzpN on spore viability.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Visible spores after development as a percent of input cell number</th>
<th>Detergent-resistant spores as a percent of total spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>133 ± 32</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>bzpN&lt;sup&gt;-&lt;/sup&gt;</td>
<td>183 ± 8</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>bzpN&lt;sup&gt;-&lt;/sup&gt; / act15::BzpN-GFP</td>
<td>163 ± 31</td>
<td>74 ± 8</td>
</tr>
</tbody>
</table>

10<sup>7</sup> cells were allowed to develop on filter pads and spores were collected in buffer by repeated washing of the filter pad using a pipette. The density of visible spores in buffer was determined by hemocytometer and the total number of collected spores was calculated. Spores were then treated with detergent, and serial dilutions of detergent-treated spores were plated on SM/5 plates in association with bacteria. The number of resultant plaques in the bacterial lawn was used to calculate the total number of detergent resistant spores as a percent of input cell number. Values are the mean ± SEM from three independent experiments.
of bacteria, I plated serial dilutions of wild-type and \(bzpN^-\) cells mixed with \(K.\ aerogenes\) bacteria on SM/5 plates and measured the diameter of well-spaced plaques daily. No significant differences were observed between the sizes of wild-type and \(bzpN^-\) plaques (Figure 14), suggesting that AprA and CfaD facilitate the expansion of colonies in a manner independent of BzpN.

Conditioned media from wild type, \(aprA^-\), or \(cfaD^-\) cells induce nuclear localization of BzpN-GFP

Basic leucine zipper transcription factors are known to translocate to the nucleus in response to extracellular signals in \(Dictyostelium\) (Huang et al., 2006). To test whether AprA, CfaD, or conditioned media from high density cells are sufficient for nuclear translocation of BzpN, I added rAprA, rCfaD, both proteins, or conditioned medium from high density cells to low density cells expressing BzpN-GFP and examined localization of the BzpN-GFP fusion protein. In low-density cells, the fusion protein showed a punctate localization that did not co-localize with either mitochondria or lysosomes (Figure 15). The addition of AprA, CfaD, or both proteins resulted in no change in BzpN-GFP localization. However, the addition of conditioned medium from high-density wild-type, \(aprA^-\), or \(cfaD^-\) cells resulted in a nuclear localization of BzpN-GFP as seen by colocalization with DAPI staining (Figure 16). These results suggest that a factor present in conditioned media from high-density cells that is not AprA or CfaD mediates localization of BzpN to the nucleus.
Figure 14: BzpN does not affect colony expansion on lawns of bacteria. Log phase cells were mixed with K. aerogenes bacteria and plated on SM/5 plates, and the size of clonal colonies was measured daily. Values are mean ± SEM, n = 2. Absence of error bars indicates that the error is smaller than the plot symbol.
Figure 15: BzpN-GFP shows a punctate localization and does not colocalize with mitochondria or lysosomes.  (A) Log phase BzpN-GFP-expressing cells were stained with Mitotracker (Invitrogen) dye, and then fixed and imaged.  (B) Log-phase BzpN-GFP expressing cells were stained with Lysotracker (Invitrogen) dye and imaged live.  Scale bars are 10 μm.
Figure 16: BzpN-GFP fusion proteins show a punctate localization at low density and a nuclear localization in response to conditioned media from high-density cells. BzpN-GFP expressing cells at low density were collected by centrifugation and resuspended in either the original media in which they had been growing (A) or conditioned media from Wild-type cells (B), aprA− cells (C), or cfaD− cells (D). Cells were incubated in shaking culture overnight, and then a sample of the culture was allowed to settle on glass chamber slides for 30 minutes. The cells were subsequently fixed, stained with DAPI, and imaged. Arrows indicate nuclear BzpN-GFP signal. (E,F) The left panels of frames A and B, respectively, were pseudocolored to highlight nuclear BzpN accumulation in the presence, but not the absence, of conditioned media from high-density cells. Colors at the red end of the spectrum indicate high BzpN-GFP intensity. Scale bar is 20 μm.
BzpN prevents proliferation of cells in the presence of conditioned media from high-density cell cultures

Conditioned media from cells at stationary density prevents proliferation, and this inhibition may depend on an unidentified small molecule present in conditioned media (Yarger et al., 1974). As BzpN negatively regulates proliferation and BzpN-GFP localizes at the nucleus in response to conditioned media from high-density cells, I tested whether BzpN was necessary for this inhibition of proliferation. When conditioned media from stationary cells was added to wild-type cells at low cell density, I saw no increase in cell density after 24 hours (Figure 17). However, under the same conditions, $bzpN^-$ cells showed a statistically significant increase in cell density, and this increase was not observed in $bzpN^-/act15::bzpN$-GFP cells. These results indicate that a factor or factors present in stationary conditioned medium prevents cell proliferation and that BzpN is necessary for the full activity of this factor.

Discussion

AprA and CfaD are autocrine signals that inhibit proliferation, though little is known regarding their mechanism of action. I provide evidence here showing that the putative transcription factor BzpN is necessary for some of the responses to AprA and CfaD. $bzpN^-$ cells proliferate rapidly during exponential growth, as would be predicted for cells lacking downstream effectors of AprA or CfaD. However, in contrast to $aprA^-$ or $cfaD^-$ cells, $bzpN^-$ cells do not proliferate to a higher stationary density than wild-type cells. This suggests that AprA and CfaD may signal through a branched pathway, of which one branch functions to inhibit proliferation at lower densities and the other at
Figure 17: *bzpN* cells but not wild-type cells proliferate in the presence of conditioned medium from cells at high density. Log phase cells were collected by centrifugation and resuspended at 0.5 x 10^6 cells/mL in conditioned media from wild-type cells grown to a density of 20 x 10^6 cells/mL. Cell densities were measured after 24 hours in shaking culture. Values are mean ± SEM (n = 5). The differences in cell densities between *bzpN* and either wild-type or *bzpN* / *act15::bzpN-GFP* cells are significant (p < 0.05, repeated measures ANOVA, Tukey’s test).
higher densities (Figure 18A). The BzpN-independent branch may not be active in low-density cells, which would explain the inability of high-density conditioned media to arrest the proliferation of low-density \textit{bzpN}^{-} cells (Figure 17). This model would predict the existence of mutants that proliferate like wild type cells at lower densities, but reach higher stationary densities than wild type. Mutants that show this phenotype have in fact been characterized (Souza et al., 1998).

Overexpression of BzpN-GFP in the wild type background resulted in a reduced maximum cell density as compared to wild type, whereas expression of BzpN-GFP in the \textit{bzpN}^{-} background resulted in a maximum cell density like wild type, suggesting that the native transcriptional or post-transcriptional regulation of \textit{bzpN} strongly affects density-dependent inhibition of proliferation. This effect could be due to a density-dependent increase in BzpN expression, which would not occur in the \textit{bzpN}^{-} / \textit{bzpN-GFP} strain, though further work is needed to evaluate this possibility. Overexpression of BzpN-GFP in \textit{aprA}^{-}, \textit{cfaD}^{-}, or \textit{qkgA}^{-} cells did not cause a significant reduction in cell density, indicating that these proteins are required for the inhibition of proliferation by BzpN. Additionally, these results strongly suggest that AprA, CfaD, and QkgA regulate BzpN activity by a mechanism other than transcriptional regulation, as BzpN-GFP is expressed in these cell lines but proliferation is not inhibited.

Intriguingly, I saw that, although rAprA and rCfaD inhibited the proliferation of wild-type cells, rAprA and rCfaD increased the proliferation of \textit{bzpN}^{-} cells, though only the increase in response to rCfaD was statistically significant. This result suggests the
Figure 18: Models for BzpN signal transduction.  (A) A BzpN-independent pathway that inhibits proliferation may be inactive at low density and active at high density. The activity of this pathway could explain the observation that \( bzpN^- \) cells do not reach higher cell densities than wild-type cells.  (B) A branched pathway may function to attenuate proliferation inhibition by a minor, proliferation-promoting activity. The existence of such a pathway could explain why \( bzpN^- \) mutants show increased proliferation in response to CfaD and AprA, if this proliferation-promoting branch is active in \( bzpN^- \) mutants.
existence of a proliferation-promoting branch of the CfaD signal transduction pathway that serves to attenuate the effect of the pathway on proliferation and that BzpN is not a component of this branch (Figure 18B). In bzpN− cells, the proliferation-promoting element of the pathway appears to still be active, thereby resulting in an increase of proliferation in response to AprA and CfaD.

I observed that BzpN-GFP fusion proteins showed a punctate intracellular localization at low cell density, and addition of conditioned media from high-density cells resulted in a nuclear localization of BzpN-GFP, indicating that an extracellular signal present at high cell density induces a localization of BzpN at the nucleus. However, this localization occurred in response to conditioned media from either aprA− or cfaD− cells, and did not occur in response to rAprA, rCfaD, or both proteins. These results suggest that an extracellular signal that is not AprA or CfaD is required for the nuclear translocation of BzpN. AprA and CfaD may activate BzpN by a mechanism independent of translocation, such as an activating phosphorylation that does not regulate subcellular localization, as is the case for the Arabidopsis bZIP transcription factor TRAB1 in response to the hormone abscisic acid (Kagaya et al., 2002). Alternatively, a low basal level of nuclear BzpN may be permissive in regards to AprA and CfaD signaling, perhaps by allowing the expression of a component of the AprA/CfaD signal transduction pathway. In this model BzpN could be activated independently of AprA or CfaD, but still could be necessary for AprA and CfaD function.
It is unclear why BzpN has been selected for during the evolution of *Dictyostelium*. *aprA*− and *cfaD*− cells show defects in spore generation (Bakthavatsalam et al., 2008; Brock and Gomer, 2005) and in the expansion of colonies on solid substrates (Phillips and Gomer, 2010), correlating the loss of these genes with a lack of fitness under some circumstances. However, *bzpN*− cells do not share these phenotypes, and thus BzpN likely functions to increase fitness in some other context. Intriguingly, expression of BzpN increases approximately 15-fold during the transition from growth to development (Rot et al., 2009), suggesting some role for BzpN during the developmental stage. BzpN may play a role in mitigating cellular stress, as cells lacking BzpN show aberrant proliferation under conditions of nitrosative or thermal stress (Phillips et al., 2011). Alternatively, BzpN may be functionally redundant with another protein affecting spore development or colony expansion, with the redundancy providing increased robustness.

Together, our data indicate that BzpN is necessary for the inhibition of proliferation by the autocrine signals AprA and CfaD, but that AprA and CfaD function in other processes that do not require BzpN. An important but unanswered question is how the putative transcription factor activity of BzpN might be altering gene expression to regulate cell proliferation. The tractability of *Dictyostelium* genetics may be useful in identifying potentially conserved genes that are upregulated or downregulated in response to BzpN activity that function to regulate proliferation, which may reveal novel approaches to reduce the proliferation of cancers.
Chapter 4. AprA functions as an endogenous chemorepellant

Introduction

Chemotaxis, or directed movement in response to a chemical gradient, is an ancient and critical behavior of eukaryotic and prokaryotic cells (Porter et al., 2011). In eukaryotes, chemotaxis proceeds through extracellular signal sensing and polarization of the actin cytoskeleton, resulting in cellular extensions (pseudopods) that facilitate movement (Rappel and Loomis, 2009). Many chemoattractants, or signals that cells move towards, have been identified (Barkley, 1969; Bleul et al., 1996; Ward, 1967; Yoshimura et al., 1989), and many molecular components involved in directional sensing and regulation of actin dynamics have been characterized (Franca-Koh et al., 2006). An alternative chemotactic process involves chemorepellants, signals that cells move away from. Some chemoattractants, such as SDF-1 and interleukin-8, act as chemorepellants at high concentrations (Poznansky et al., 2000; Tharp et al., 2006), and the protein semaphorin III acts as a chemorepellant in the context of neuronal growth cone guidance (Messersmith et al., 1995). Chemorepellants may function in the resolution of inflammation (Vianello et al., 2005), gastrulation (Yang et al., 2002), the pathogenicity of the parasite Entamoeba histolytica (Zaki et al., 2006) and metastasis (Bagci et al., 2009). However, few endogenous chemorepellants have been identified, and relatively little is known regarding their mechanism of action.

1 The work in this chapter has been published (Phillips and Gomer, 2012).
The eukaryote *Dictyostelium discoideum* is an excellent model for the study of chemotactic processes. In the presence of nutrients, *Dictyostelium* exists as unicellular amoebae that reproduce by fission. When starved, cells secrete and respond to the chemoattractant cAMP, leading to the aggregation of cells and the formation of fruiting bodies (Kessin, 2001). *Dictyostelium* chemotaxis toward cAMP involves G protein-coupled receptors (Klein et al., 1988), heterotrimeric G proteins (Brzostowski et al., 2004; Kumagai et al., 1989), Ras (Chubb et al., 2000; Reymond et al., 1986) PI3-kinase (Funamoto et al., 2001), phospholipases (Bominaar and Van Haastert, 1993), and other proteins (Booth et al., 2005; Franca-Koh et al., 2006). There also appear to be *Dictyostelium* chemorepellants. For instance, when a small spot of cells is placed adjacent to a much larger spot, cells in the small spot tend to move away from the larger spot (Kakebeeke et al., 1979), and when two spots of high-density cells are placed next to each other, cells show a greater degree of movement away from the adjacent spot than toward it (Keating and Bonner, 1977). In addition, the synthetic cAMP analog 8CPT-cAMP induces negative chemotaxis through localized phospholipase C inhibition (Keizer-Gunnink et al., 2007). However, no endogenous chemorepellants have been identified.

Interestingly, although *aprA* cells are able to chemotax towards cAMP, as evidenced by the ability of the cells to aggregate, and *aprA* cells show random motility like that of wild-type cells, colonies of *aprA* cells show a reduced rate of expansion compared to wild type (Phillips and Gomer, 2010). One explanation for this failure of colonies to expand is that wild-type but not *aprA* cells produce an autocrine
chemorepellant that is high in concentration at high cell density, and forms a gradient towards regions of low cell density, facilitating the spreading of cells. In this chapter I provide evidence indicating that AprA functions as an autocrine chemorepellant for vegetative Dictyostelium cells.

Materials and Methods

Insall chamber assay

To measure the effect of AprA on cell displacement using an Insall chamber (Muinonen-Martin et al., 2010), cells in shaking culture were diluted to $5 \times 10^4$ cells/mL in HL5 media, and then 300 µL volumes of the dilution were grown on 22 x 22 mm glass coverslips for 1 hour at 20°C. I then utilized an Insall chamber slide, a kind gift from Robert Insall, consisting of two concentric square depressions separated by a bridge (Muinonen-Martin et al., 2010). Both depressions and the bridge were filled with HL5 media, and then the media was removed from the coverslips, which were then placed face down on the chamber. Media was then removed from the outer chamber and was replaced by either rAprA in HL5 or HL5 alone. Cells located on the bridge between the square depressions were then filmed using a 10X objective. After an initial 20-minute period, the displacement of at least 10 individual cells per experiment was measured over a period of one hour. The displacement of cells in the direction away from the rAprA source was then calculated by vector decomposition. To analyze cell displacement in the absence of rich media, cells were grown on coverslips for one hour as described above, then media was removed and cells were washed twice with 300 µL PBM (20 mM KH$_2$PO$_4$, 0.01 mM CaCl$_2$, 1 mM MgCl$_2$, pH 6.1, with KOH). The Insall chamber assay
was then performed as described above, except that PBM was used in place of HL5 for all steps.

**Measurement of cell displacement at colony edges**

To measure cell displacement at the edge of a colony, 10 μL of cells at 2-4 x 10^6 cells/mL was pipetted as a spot at the center of a well in a 2-well glass chamber slide (Nunc) and cells were allowed to settle for 15 minutes. Media and unadhered cells were then removed by pipetting and the chamber was filled with 1 mL of HL5 media and placed on an inverted microscope. After 1 hour, cells at the edge of the colony were filmed using time-lapse video microscopy with a 4X objective for a period of five hours using a Panasonic WV-BL200 monochrome CCTV camera and a Panasonic AG-6740 AVHS time lapse video recorder. Following filming, the movement of at least 10 randomly chosen individual cells per video was followed for the five hour period, the distance and angle between the starting and ending point for individual cells was measured to obtain a displacement vector, and the displacement of individual cells in the direction outwards from the colony was found by calculating the displacement vector component in the direction away from the cell colony (vector decomposition). A small percentage of filmed cells (< 5%) showed no significant movement during filming; these cells were judged to be dead and were not measured. Imaging of *Dictyostelium* cell colonies grown with *K. aerogenes* was done as described previously (Phillips and Gomer, 2010).
**Under-agarose chemotaxis assay**

To determine the effect of AprA or CfaD on the directionality of cell movement, cells from axenic shaking culture were diluted to $1 \times 10^4$ cells/mL in HL5, and 10 ml was added to a 100 x 20 mm tissue culture dish (Falcon). After 10 minutes, the media and unadhered cells were decanted, and 10 ml of fresh media was added to the dishes, which were then incubated overnight at 20°C. A solution of 1% low-EEO molecular biology-grade agarose (Fisher) in SM media (in 1 L ddH$_2$O: 10 g glucose, 10 g proteose peptone, 1 g yeast extract, 1 g MgSO$_4$*7H$_2$O, 1.9 g KH$_2$PO$_4$, 0.6 g K$_2$HPO$_4$, pH 6.2) was then prepared by microwaving, and 20 ml volumes of this solution were poured into 100 x 20 petri plates and allowed to solidify. The media from the culture dishes was then decanted and replaced with the solidified agarose, and a 2 x 44 mm rectangular hole was cut into the center of the agarose. The culture dish was then placed on an inverted microscope, and 200 μL of HL5 media, HL5 with rAprA at 2000 ng/mL, or HL5 with rCfaD at 1000 ng/mL was added to the hole. A 4X objective was used to film cells underneath the agarose approximately 1.5 mm away from the hole. After 30 minutes, the displacement of at least 10 randomly chosen cells was measured over a period of 2 hours, and the displacement of cells in the direction away from the hole was calculated by vector decomposition. For analysis of cell displacement adjacent to a source of conditioned media, cells of the indicated genotype were grown in HL5 media in shaking culture to $1 \times 10^7$ cells/mL, and then cells were collected by centrifugation at 800 x g for 3 minutes. The supernatant was then collected and passed through a 0.45-micron filter (VWR). To dialyze the conditioned media, a 400 μL volume was injected into a 10-kDa cutoff dialysis cassette (Thermo Scientific) and dialyzed against 400 mL of HL5 media.
overnight. The under-agarose assay was performed as described above, except that a 200 μL volume of dialyzed conditioned media was used to fill the hole in the agarose.

**Measurement of cell displacement in a uniform concentration of AprA**

Wild-type cells in shaking culture were diluted to 1 x 10^5 cells/mL in HL5, and then 200 μL of this dilution was added to a well of an 8-well glass chamber slide (Nunc). After 5 minutes, media and unadhered cells were removed and replaced with 200 μL of fresh HL5 media. Cells were incubated at room temperature for 30 minutes, and then the media was removed and replaced with 200 μL of 2 μg/mL rAprA in HL5 or HL5 with an equivalent volume of buffer, and cells were then filmed using a 10X objective. After an initial 20-minute period, the displacement (the scalar distance between the starting and ending positions) of randomly chosen individual cells over the course of two hours was measured.

**Imaging of Lifeact-GFP and MyoII-GFP-expressing cells in a gradient of rAprA**

Ax2 cells transformed with extrachromosomal vectors encoding Lifeact-GFP (a gift from Dr. David Knecht, University of Connecticut) or MyoII-GFP (Moores et al., 1996) were used in the Insall chamber assay as described above. 30 minutes after the experimental setup, individual cells were tracked for 5 minutes to determine the direction of cell movement, and then GFP localization was immediately imaged using a 40X objective.
Assaying the chemorepellant activity of rAprA on starved cells

Cells in axenic shaking culture at 1-4 x 10^6 cells/mL were washed twice and resuspended in Na/KPO₄ starvation buffer (2.5mM Na₂HPO₄, 9.5 mM KH₂PO₄, pH 6.1) at 5 x 10^6 cells/mL in a 5 mL volume and incubated for 5 hours at 20˚ C in shaking culture. Cells were then diluted to 5 x 10^4 in Na/KPO₄ buffer, and 300 µL of this dilution was pipetted onto a 22x22 mm coverslip in a humid box. Cells were allowed to settle for 30 minutes, and then cells were assayed for chemorepulsion in response to rAprA using an Insall chamber as described previously, with a 2000 ng/mL solution of rAprA in Na/KPO₄ buffer.

Imaging of CRAC-GFP-expressing cells in a gradient of rAprA

Cells expressing CRAC-GFP were taken from axenically growing shaking culture at 1-4 x 10^6 cells/mL, resuspended in PBM, and incubated in shaking culture for 2 hours to reduce an autofluorescent background signal present in growth medium. Cells were then diluted to 5 x 10^4 cells / mL in PBM and were situated in a gradient of rAprA using an Insall chamber as described previously, using a 2000 ng/mL solution of rAprA in PBM. 30 minutes after setup of the gradient, individual cells were tracked for a 5-minute period, and then the GFP signal was immediately imaged using a 60X objective.

Cell tracking and cell track analysis

To obtain cell tracks, videos of cells in a gradient of AprA were made using an Insall chamber as described above. Videos of an hour in length were processed to yield a TIFF stack of 104 images, which was then used to track randomly chosen individual cells using
the ImageJ manual tracking plugin (Sheffield, 2007). The track data was then imported into the “Chemotaxis and migration tool” plugin (Ibidi) to calculate forward migration index, cell speed, directionality, and Rayleigh test. To examine the persistence of cell movement and to create a histogram of 35-second interval displacements, Δx values obtained for all intervals during cell tracking (which correspond to the displacement along the direction of the rAprA gradient) were analyzed using Microsoft Excel and Prism (GraphPad Software, San Diego, CA).

Results

Wild-type but not aprA− or cfaD− cells show directed movement away from regions of high cell density

I previously found that colonies of aprA− and cfaD− cells expand less rapidly than wild-type cells on a lawn of bacteria, although these mutants show no difference in random cell motility at low cell density as compared to wild type (Phillips and Gomer, 2010). To test whether wild-type cells show a directed movement away from areas of high cell density, I established small colonies of wild-type cells in growth media and measured the displacement of individual cells at the colony edges by video microscopy. These cells showed an average displacement away from the cell colony (Figure 19), with 94 ± 6% (mean ± SEM, n = 3, at least 10 cells per experiment) of measured cells showing a displacement away from the colony. For aprA− or cfaD− cells, although cells were motile, the average displacement of cells was not strongly biased in any direction, with 50 ± 9% of aprA− cells and 67 ± 4% of cfaD− cells showing displacement away from the colony. These results show that wild-type but not aprA− or cfaD− cells show a directed
Figure 19: Wild type, but not *aprA*− or *cfaD*− cells, show a directed movement away from a colony of cells. A spot of cells was allowed to settle in a cell culture chamber, and then the chamber was filled with media. The edge of the colony was filmed, and the movement of individual cells was followed over a period of five hours. The average displacement of cells in the direction away from the colony is shown. Values are mean ± SEM, n = 3, with the displacement of at least 10 cells per genotype measured for each independent experiment. The difference between wild type and either *aprA*− or *cfaD*− is significant (p < 0.001, 1-way ANOVA, Tukey’s test).
movement away from regions of high cell density, indicating that AprA and/or CfaD are
required for directed movement away from a cell colony.

**Cells show directed movement away from a source of AprA but not CfaD**

One explanation for the directed movement of wild-type cells, but not $aprA^{-}$ or
$cfaD^{-}$ cells, away from a colony is that AprA and/or CfaD may be acting as an autocrine
chemorepellant, present at high concentrations where cell density is high and facilitating
the spreading of cells. To test this possibility, I used an under-agarose assay (Woznica
and Knecht, 2006), which consists of placing a layer of agarose over a uniform field of
cells, adding rAprA or rCfaD to a well in the agarose, and examining the displacement of
individual cells adjacent to the well. When media alone was added to the well, cells
adjacent to the well showed no significant bias in displacement in the direction away
from the well (Figure 20), with $56 \pm 4\%$ of cells showing displacement away from the
well. However, when rAprA was added to the well, cells showed an average
displacement away from the well, and $73 \pm 9\%$ of cells moved away from the well. When
rCfaD was added to the well, no significant bias in displacement was observed. I
examined actin and myosin localization in cells in a rAprA gradient and invariably
detected actin localization at the leading edge and myosin II localization at the trailing
edge (Figure 21), suggesting that cells that show directed movement away from rAprA
move with the classical mechanism of actin-rich protrusions at the leading edge of the
cell and myosin II-mediated contraction at the rear (Rappel and Loomis, 2009). These
results indicate that AprA functions as a chemorepellant and suggest that CfaD does not
share this function. Since rAprA and rCfaD were produced in identical protocols, and
**Figure 20: rAprA but not rCfaD repels cells.** A population of wild-type cells was established under a layer of agarose, then a well was cut in the agarose and either buffer, rCfaD or rAprA was added. Cells adjacent to the well were filmed, and the displacement of individual cells in the direction away from the well over 2 hours was measured. Values are mean ± SEM, n ≥ 4, with the displacement of at least 10 cells per genotype measured for each independent experiment. The differences between the displacements for AprA and either buffer or CfaD are significant (p < 0.01, one-way ANOVA, Dunnett’s test).
Figure 21: Cells moving towards or away from an AprA source show F-actin at the leading edge and myosin II at the rear. Cells expressing the indicated protein were placed in a gradient of rAprA (with the source located to the right of all imaged cells) using an Insall chamber, and tracked for five minutes to determine the direction of cell movement, which is indicated by a white arrow. GFP localization was then imaged using a 40X objective. Bar is 10 microns. For GFP-myosin II, images are combined GFP and phase contrast channels.
rCfaD does not have chemorepellant activity, the chemorepellant activity of AprA is likely not due to a contaminant from protein expression and purification.

The extracellular concentration of AprA increases as a function of cell density in shaking culture and reaches a maximum concentration of 300 ng/mL (Choe et al., 2009). To test the effect of different concentrations of rAprA on cell movement, I used an Insall chamber assay (Muinonen-Martín et al., 2010), which is a more tractable assay for chemotaxis than the previously described under-agarose assay. I tested the response of cells to the chemoattractant folate and to rAprA and observed chemotaxis towards folate and away from rAprA (Figure 22), showing that cells used in this assay are chemotaxis competent and chemorepulse from rAprA. A source of rAprA at a concentration of 20 ng/mL was sufficient for chemorepellant activity (Figure 23). These results indicate that the chemorepellant effect of AprA occurs at physiological concentrations.

A chemorepellant activity present in dialyzed conditioned media from wild-type cells is reduced in aprA^-conditioned media

If AprA functions as a chemorepellant, then conditioned media from wild-type cells should have chemorepellant activity, and this activity may be reduced or absent in conditioned media from aprA^-cells. To test this prediction, I measured the displacement of cells adjacent to conditioned media from wild-type cells, conditioned media from aprA^-cells, or fresh media using the under-agarose assay. Conditioned media from both wild-type and aprA^-cells at high density had a chemorepellant effect (Figure 24). However, when conditioned media was dialyzed against fresh media using a 10 kDa
Figure 22: Cells in an Insall chamber move towards a folate source and away from a rAprA source. Cells adjacent to a source of folate, rAprA, or HL5 media in an Insall chamber were filmed for one hour, and the displacement of individual cells relative to the source was measured. Positive values indicate movement towards the source. Values are mean ± SEM from at least 3 independent experiments, with at least 10 cells measured for each experiment. For both rAprA and folate conditions, the differences in average displacement as compared to the HL5 media condition are significant (p < 0.05, t-test). The difference between HL5 media and the value 0 is not significant (paired t-test).
Figure 23: The chemorepellant activity of AprA is dosage dependent. Wild-type cells adjacent to a source of rAprA at the indicated concentration in HL5 media were filmed for one hour, and the displacement of individual cells away from the rAprA source was measured. The differences in average displacement are significant between either 20ng/mL or 2000 ng/mL conditions and the condition without rAprA (p <0.05, t-test). Values are mean ± SEM, n ≥ 3.
Figure 24: Undialyzed conditioned media from WT and aprA\(^{-}\) cells has chemorepellant activity. Undialyzed conditioned media from high-density cells was tested for chemorepellant activity on wild-type cells using the under-agarose assay described in Figure 2. The average cell displacement relative to the conditioned media source is shown, with positive values indicating movement away from the conditioned media source. Values are mean ± SEM, n \(\geq\) 2, with at least 10 cells measured for each experiment. The values for both wild-type and aprA\(^{-}\) conditioned media are significantly different from a value of 0 (p < 0.05, paired t-test) and are not significantly different from each other (t-test).
cutoff membrane and then used in the under-agarose assay, conditioned media from wild-type cells caused a significant bias in cell displacement away from the conditioned media as compared to fresh media (Figure 25), whereas conditioned media from aprA⁻ cells did not show a significant effect on cell displacement. These results indicate that something larger than 10kD that is present in wild-type conditioned media but not significantly present in aprA⁻ conditioned media functions as a chemorepellant, supporting the hypothesis that AprA itself is a chemorepellant. In addition, a chemorepellant that is smaller than 10 kDa is present in high-density aprA⁻ conditioned medium.

**AprA shows chemorepellant activity in the absence of rich media**

An autocrine chemorepellant could potentially work by degrading or inactivating a chemoattractant present in the surrounding area, causing the chemoattractant concentration to be low in areas of high cell density and high elsewhere. This would then create a gradient of the chemoattractant, causing cells to move away from areas of high cell density. As at least one compound in rich media, folate, functions as a Dictyostelium chemoattractant (Pan et al., 1972), this model of chemorepulsion could potentially be occurring under our experimental conditions. I thus examined whether rich media is necessary for AprA chemorepellant activity by assaying for chemorepellant activity using cells in buffer. Under these conditions, I still observed a chemorepellant effect of rAprA that was not significantly different from that observed in rich media (t-test; Figure 26 and 22); 48 ± 5% of cells measured showed movement away from buffer (with buffer also in the opposite chamber), whereas 81 ± 4% of cells showed movement away from rAprA in buffer (p < 0.01, t-test, n = 3). These results show that the chemorepellant effect of AprA
Figure 25: Cells are repelled by conditioned media from wild-type but not aprA\textsuperscript{−} cells. Under-agarose assays were done as described previously using dialyzed conditioned media. Values are mean ± SEM, n = 3, with the displacement of at least 10 cells per genotype measured for each independent experiment. The displacements for WT conditioned media are significantly different from both fresh media and aprA\textsuperscript{−} conditioned media (p < 0.01, one-way ANOVA, Tukey’s test). The difference between aprA\textsuperscript{−} conditioned media and the value 0 is not significant (paired t-test).
Figure 26: AprA functions as a chemorepellant in the absence of rich media. Cells were placed adjacent to a source of rAprA or buffer using an Insall chamber and filmed for one hour. The displacement of individual cells in the direction away from the rAprA source was then measured. Values are mean ± SEM, n = 3. The difference between the indicated conditions is significant (p < 0.05, one-tailed t-test). The 5 hour starved WT condition is not significantly different from the value 0 (p > 0.05, paired t-test).
does not require rich media, strongly suggesting that AprA does not function as a chemorepellant by inactivating a chemoattractant.

**AprA shows no detectable chemorepellant activity on starved cells**

AprA is most strongly expressed during vegetative growth, though expression is also detected during aggregation and development (Brock and Gomer, 2005). To examine whether AprA affects the movement of cells at the aggregation stage, I starved wild-type cells for 5 hours in buffer to induce the transition from growth to development, and then assayed the chemorepellant effect of rAprA on these cells. Under these conditions, no bias in the direction of cell movement was evident (Figure 26). This result suggests that rAprA does not act as a chemorepellant for cells during the aggregation stage.

**The chemorepellant effect of AprA requires CfaD, QkgA, and Gα8, but not BzpN, PLC, or PI3-kinases 1 and 2**

The G protein Gα8, the kinase QkgA, and the putative transcription factor BzpN are necessary for the proliferation-inhibiting activity of AprA (Bakthavatsalam et al., 2009; Phillips and Gomer, 2010; Phillips et al., 2011), implicating these proteins in AprA signaling. Additionally, the secreted protein CfaD is necessary for AprA activity (Bakthavatsalam et al., 2008). To test whether these proteins are necessary for the chemorepellant activity of AprA, I examined whether cell lines mutant for these proteins were repelled by rAprA in an Insall chamber assay. I saw that wild-type cells adjacent to a source of rAprA showed a significant bias in displacement away from rAprA (Figure 27). Similarly, aprA− and bzpN− cells showed directed movement away from a rAprA
Figure 27: AprA requires the proteins Gα8, QkgA, and CfaD but not BzpN or PLC for activity. Cells in HL5 media were placed adjacent to a source of rAprA or media using an Insall chamber and filmed for one hour. The displacement of individual cells in the direction away from the rAprA source was then measured. “*” indicates p < 0.05, “**” p < 0.01, and “***” p < 0.001 (t-test). The differences in average displacement between genotypes that show a significant chemorepellant response are not significant (one-way ANOVA, Tukey’s test).
source. In contrast, \( \alpha 8^- \), \( k g A^- \), and \( c f a D^- \) cells showed no significant bias in displacement relative to a rAprA source. These results indicate that \( \alpha 8 \), QkgA, and CfaD are necessary for the chemorepellent effect of AprA, whereas BzpN is not. Consistent with our interpretation of AprA chemorepellent activity acting to disperse cell colonies, I found that, like \( aprA^- \), \( c f a D^- \), and \( k g A^- \) cells, \( \alpha 8^- \) cells showed a reduced spreading at the edge of cell colonies as compared to wild type, whereas \( b z p N^- \) cells did not (Figure 28).

The synthetic cAMP analog 8CPT-cAMP acts as a chemorepellant for starved \textit{Dictyostelium} cells, and this chemorepulsion requires phospholipase C (PLC) and the activity of PI3-kinases 1 and 2 (Keizer-Gunnink et al., 2007). I observed that both \( p l c^- \) and \( p i 3 k 1^-2^- \) cells showed movement away from a rAprA source in a manner similar to wild-type cells (Figure 27). I further examined the role of PI3K activity in AprA-mediated chemorepulsion by using cells expressing CRAC-GFP (for \textit{Cytosolic Regulator of Adenylyl Cyclase}), a marker for PI3K activity, which localizes to the upgradient side of a cell during chemotaxis towards cAMP (Parent et al., 1998). When situated in a gradient of rAprA, cell tracking over a 5 minute period revealed that 54% of cells expressing CRAC-GFP moved down the rAprA gradient, 27% of cells moved up the gradient, and 19% of cells moved roughly parallel to the gradient, suggesting that rAprA has a chemorepellent effect on these cells. When CRAC-GFP was imaged, the majority of cells showed cytosolic CRAC-GFP localization, and localization did not correlate with the direction of the rAprA gradient for the whole cell population or for the subset of cells moving down the rAprA gradient (Figure 29). This result indicates that PLC and PI3K
Figure 28: $ga8^-$ cell colonies phenocopy $aprA^-$ cell colonies. Small spots of *Dictyostelium* cells were grown in glass chamber slides with *K. aerogenes* overnight, and colony edges were imaged the following day. Scale bar is 100 microns.
Figure 29: CRAC-GFP localization in the presence of a rAprA gradient. CRAC-GFP-expressing cells in PBM were situated in a gradient of rAprA using an Insall chamber, filmed to determine the direction of cell movement, and imaged by fluorescence microscopy. The higher concentration of rAprA is at the right of the image, and the arrows indicate the direction of cell movement preceding fluorescence imaging. No signal was detected when using wild-type cells with identical microscopy conditions. Scale bar is 10 μm.
activity are not necessary for the chemorepellant activity of AprA, and that AprA and 8CPT-cAMP function as chemorepellants through distinct mechanisms.

**AprA biases the direction of cell movement but does not affect average cell speed**

To gain insight into the mechanism by which AprA might affect cell movement, I tracked the movement of wild-type cells in a gradient of rAprA over an hour and examined the parameters of cell movement. Whereas no bias in the direction of movement was evident for a media control (Figure 30A), cells tended to move away from the rAprA source, and the center of mass of the cell end points indicated a bias away from the rAprA source (Figure 30B). Tracks of cells in a gradient of rAprA showed a negative forward migration index (FMI) that was significantly different from the FMI for the media control (Table 9), supporting the interpretation of AprA as a chemorepellant. The average speed of cells in a gradient of rAprA was not significantly different from the control (Table 9) and was consistent with previous measurements of randomly motile vegetative cells (Hoeller and Kay, 2007; Tuxworth et al., 1997), indicating that AprA does not stimulate an increase in the average speed of cells. Supporting this conclusion, cells in a uniform concentration of 2 µg/mL rAprA showed a displacement of 152 ± 33 µm over an interval of 2 hours, whereas cells in media showed a displacement of 157 ± 19 µm; the difference is not significant. Cells in a gradient of rAprA showed a significantly higher directionality (a measure of how directed, as opposed to random, the movement of a cell is) than the control (Table 9), further indicating that AprA affects the direction of cell movement. Finally, I examined whether the distribution of the endpoints of cell tracks was nonrandom by performing a Rayleigh test. The distribution for cell
**Figure 30: Tracking of cells adjacent to an AprA source.** Wild-type cells in the absence (A) or presence (B) of a rAprA gradient were filmed using an Insall chamber, cells were tracked over an hour-long period, and tracks were graphed. The AprA source is on the right side of the origin. Red dots represent the final position of cells, and green dots are the averaged center of mass for all tracks. The tracks are a compilation of three independent experiments with at least seven tracks per experiment.
Table 9: The effect of AprA on forward migration index (FMI), cell speed, cell directionality, and cell distribution.

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<thead>
<tr>
<th></th>
<th>Media control</th>
<th>rAprA</th>
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<tbody>
<tr>
<td>FMI</td>
<td>-0.01 ± 0.04</td>
<td>-0.18 ± 0.06*</td>
</tr>
<tr>
<td>Speed, μm/min</td>
<td>4.9 ± 0.2</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>Directionality</td>
<td>0.21 ± 0.03</td>
<td>0.34 ± 0.03***</td>
</tr>
<tr>
<td>Rayleigh test (p value)</td>
<td>0.52</td>
<td>0.003</td>
</tr>
</tbody>
</table>

The data from Figure 30 was analyzed to calculate the forward migration index (a measure of cell movement in the direction of a gradient in respect to total cell movement, with zero indicating no movement in the direction of the gradient), cell speed, directionality (the ratio of the Euclidean distance to the total distance traveled), and p values for the Rayleigh test, a test for nonrandom distributions of cell end points. “*” indicates a significant difference with p < 0.05, and “***” indicates p < 0.001 (t-test).
endpoints adjacent to a rAprA source was significantly biased, whereas the distribution of endpoints for the media control was not (Table 9). Together, these data indicate that AprA regulates the directionality of cell movement so that cells move away from high AprA concentrations, and that this regulation does not involve a change in average cell speed.

**AprA affects directionality but not persistence of cell movement**

To further characterize how AprA affects movement, I used the cell tracking data to examine how AprA might affect the directional persistence of cells. Movies of cells were processed into 35-second intervals, and the displacement of cells in the direction of the AprA source was determined for all cells and all intervals. Most commonly, cells showed no change in position over a 35-second interval (Figure 31). For the control condition, the distribution of displacements revealed no bias in movement, whereas for cells in a gradient of rAprA a bias in displacement in the direction away from rAprA was evident (Figure 31 and Table 10). To determine whether rAprA might cause an increase in cell velocity in certain directions, I then examined the average displacement for two subsets of intervals: intervals in the direction towards or away from the rAprA source. There was no significant difference in the average displacement for cells moving away from the source between the rAprA and the control condition, and there was no significant difference in the average displacement for cells moving toward the source between the two conditions (Table 10). These results suggest that AprA does not significantly increase the velocity of cells in a directional manner.
Figure 31: AprA affects the directionality of cell movement. The data from cell tracking was used to create a histogram of all measured 35-second interval cell displacements in the direction of the rAprA source, with positive values indicating movement toward the source. Histogram bins are in units of 1.63 microns, a distance that corresponds to 1 pixel on the videos used for measurement. “*” indicates a significant difference with p < 0.05, and “**” indicates a significance of p < 0.01 (one-tailed t-tests). Values are mean ± SEM from 3 independent tracking experiments.
Table 10: The effect of AprA on the direction of cell displacement and cell persistence over 35-second intervals.

<table>
<thead>
<tr>
<th>Probabilities of cell displacement over one 35-second interval:</th>
<th>Media control</th>
<th>rAprA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Displacement away from source (Pₐ)</td>
<td>0.27 ± 0.02</td>
<td>0.35 ± 0.01**</td>
</tr>
<tr>
<td>Displacement toward source (Pₜ)</td>
<td>0.28 ± 0.02</td>
<td>0.20 ± 0.02*</td>
</tr>
<tr>
<td>No displacement</td>
<td>0.46 ± 0.01</td>
<td>0.44 ± 0.01</td>
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<tr>
<th>Average displacement of indicated subset, µm:</th>
<th></th>
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<tbody>
<tr>
<td>Displacements away from source</td>
<td>3.3 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Displacements toward source</td>
<td>3.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Normalized probability of two subsequent movements in the same direction:</th>
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<tbody>
<tr>
<td>Away from source (NA2)</td>
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<tr>
<td>Toward source (NT2)</td>
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<th>Normalized probability of three subsequent movements in the same direction:</th>
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<tr>
<td>Away from source (NA3)</td>
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<tr>
<td>Toward source (NT3)</td>
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<th>Normalized probability of four subsequent movements in the same direction:</th>
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<tr>
<td>Away from source (NA4)</td>
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<tr>
<td>Toward source (NT4)</td>
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</tbody>
</table>

The data from cell tracking is shown here as the probability that in a 35-second interval, a cell will show displacement away from the rAprA source, toward the source, or no displacement. Values are mean ± SEM from 3 independent experiments. “*” indicates that the differences between control and rAprA gradient conditions are significant with p < 0.05, and “**” indicates p < 0.01 (t-test). For the control, Pₐ and Pₜ are not significantly different, whereas in a gradient of rAprA, Pₐ and Pₜ are significantly different with p < 0.01 (t-test). For all normalized probabilities of subsequent movements in the same direction, differences between the control and rAprA conditions are not significant (p > 0.05, t-test).
To determine the effect of rAprA on the persistence of cell movement, I first defined \(P_A\) as the probability that a cell would move away from rAprA, and \(P_T\) as the probability that a cell would move toward rAprA. As shown in Table 10, \(P_A\) and \(P_T\) are roughly the same for the media control, and significantly different in a gradient of rAprA. I then measured, for each cell, \(P_{A2}\), the percent of intervals where the cell moved away from rAprA in one interval and away again in the next interval. Because the probability that a cell will move away is different depending on whether there is a gradient of rAprA, I calculated \(NA_2 = P_{A2}/(P_A)^2\), or the observed probability of two subsequent movements away from rAprA divided by the expected probability that such an event would happen at random. \(NA_2\) thus represents a normalized probability that a cell will move away from rAprA in two subsequent time intervals, with higher values indicating more persistence in movement than would be expected at random. I found that \(NA_2\) was not statistically different between the rAprA gradient condition and the control, and that the corresponding \(NT_2\) (the normalized probability of a cell moving towards rAprA in two sequential 35-second intervals) was also not different between rAprA gradient and control (Table 10). I similarly calculated the normalized probability that a cell will move away from rAprA in either three or four consecutive intervals as \(NA_3 = P_{A3}/(P_A)^3\) or \(NA_4 = P_{A4}/(P_A)^4\). For both control and rAprA gradient conditions, \(NA_4\) was greater than \(NA_3\), and \(NA_3\) was greater than \(NA_2\), suggesting that longer runs of persistence occur at a higher rate than would be expected at random as compared to shorter ones. I observed no statistically significant difference in the normalized probability that a cell moved away in three or four consecutive time intervals between rAprA gradient and control conditions. Similarly, the corresponding \(NT_3\) and \(NT_4\) values are not significantly different between
rAprA gradient and control experiments. Together, these results suggest that the chemorepulsion mediated by rAprA is not due to an altered persistence in movement away from or toward the source of rAprA.

Discussion

Colonies of aprA− cells expand less rapidly than wild-type colonies, despite the fact that aprA− cells proliferate more rapidly than wild-type cells (Brock and Gomer, 2005; Phillips and Gomer, 2010). Our data support the hypothesis that rAprA functions as an autocrine chemorepellant for Dictyostelium cells, and that this chemorepellant function may facilitate the spreading of cell colonies.

I found that dialyzed conditioned media from wild-type but not aprA− cells has chemorepellant activity. However, when conditioned media was taken directly from cells, filter sterilized, and assayed, conditioned media from aprA− cells showed chemorepellant activity. This result suggests that a small molecule or molecules that have chemorepellant activity accumulate in the media of high-density cultures of aprA− cells. Alternatively, conditioned media from high-density cells may be depleted of nutrients, and using this depleted media could establish a gradient of nutrients leading away from the source, which might lead to cells moving away. Regardless, this chemorepellant effect is likely not functioning physiologically in the movement of cells away from areas of high cell density, as aprA− cells do not show a strong bias in movement away from a colony (Figure 19).
Multiple lines of evidence suggest that AprA acts as a ligand for a G protein-coupled cell surface receptor as opposed to interacting with another extracellular factor or factors (Bakthavatsalam et al., 2009; Choe et al., 2009). I found that rAprA shows chemorepellent activity in the absence of rich media, indicating that AprA does not function by modifying factors in media with chemokinetic properties. This result further suggests that AprA acts as a ligand as opposed to modifying an extracellular factor.

I found that the G protein Go8 and the kinase QkgA are necessary for the chemorepellant activity of AprA as well as its proliferation-inhibiting activity, but that the putative transcription factor BzpN is dispensable for AprA chemorepellant activity despite being necessary for inhibition of proliferation. These results suggest that AprA inhibits proliferation and induces chemorepulsion using partially overlapping signal transduction pathways, and that the signal transduction branches, with BzpN being a component of the proliferation-inhibiting pathway but not the chemorepellant pathway.

Whereas the previously reported Dictyostelium chemorepellant 8CPT-cAMP requires PLC and PI3-kinases 1 and 2 for activity, I found that these proteins are not required for the chemorepellant activity of rAprA. Further, whereas 8CPT-cAMP acts on starved cells (Keizer-Gunnink et al., 2007), rAprA shows no chemorepellant activity on starved cells. These results suggest that these chemorepellants likely function through different mechanisms, and that when cells are aggregating, the AprA chemorepulsion mechanism is disabled. I further investigated the role of PI3-kinase activity on chemorepulsion and saw that CRAC-GFP localization did not correspond to a rAprA
gradient, suggesting that polarized PI3-kinase activity is not essential for rAprA chemorepellant activity.

I tracked the movement of cells in a gradient of rAprA and found that cells showed a negative migration index and more directed movement than control cells, although the difference in average speed for cells in a rAprA gradient and control cells was not significant. I also found that AprA does not affect the persistence of cell movement in a directional manner. Taken together, our data indicate that rAprA functions as a chemorepellant by biasing the movement of cells in the direction of a decreasing AprA gradient, and not by affecting the speed of cells or the persistence of cell movement.

A Dictyostelium autocrine chemorepellant mechanism may have been selected for because it spreads out groups of vegetative cells, as cells would tend to move away from areas of high cell density by moving down an AprA gradient. This movement would result in coverage of a larger area by a population of cells and thus access to a larger quantity of nutrients, facilitating growth. Such an effect seems evident during growth of wild-type or aprA− cells on lawns of bacteria, as wild-type colonies expand and clear the bacterial lawn more rapidly than aprA− cell colonies (Phillips and Gomer, 2010). The proliferation-inhibiting activity of AprA (Brock and Gomer, 2005) may act in cooperation with this process by preventing proliferation at high cell density, and thus conserving local resources, giving high-density cells the opportunity to spread before being depleted of nutrients and undergoing aggregation.
Few endogenous chemorepellants have been identified, and their mechanisms of action are largely unknown. I have identified an endogenous chemorepellant in *Dictyostelium discoideum* and have identified conserved proteins necessary for chemorepellant function that may serve as components of a signal transduction pathway. *Dictyostelium*, a highly tractable model organism, has been immensely useful in elucidating conserved chemotactic mechanisms. This fact suggests the exciting possibility that the study of AprA and its mechanism of action could shed light on conserved mechanisms of chemorepulsion, perhaps providing insight or therapeutic approaches for disease states in which chemorepellants play a role.
Chapter 5. The kinase PakD is required for proliferation inhibition by AprA and CfaD and for the chemorepellant activity of AprA

Introduction

p21-activated kinases (PAKs) are a conserved family of kinases that bind to and are activated by small GTPases such as Rho and cdc42 (Bokoch, 2003). PAKs function to regulate actin dynamics in processes such as bud growth in Saccharomyces cerevisiae (Leberer et al., 1992), growth cone guidance in developing Drosophila neurons (Hing et al., 1999) and chemotaxis towards cAMP in Dictyostelium (Chung and Firtel, 1999; Lee et al., 2004). PAK1 induces the formation of filopodia and membrane ruffles in human fibroblasts (Sells et al., 1997), whereas Drosophila Pak3 inhibits lamellipodia formation in cell culture (Asano et al., 2009), indicating that PAKs can positively or negatively regulate actin-based structures. PAKs also regulate proliferation, and most evidence suggests that PAKs positively regulate proliferation and growth (Bokoch, 2003). In COS-1 fibroblasts, PAK1 stimulates mitogenic MAP kinase signaling (Eblen et al., 2002) and in human fibroblasts PAK2 inhibits the tumor suppressor NF2 by phosphorylation, resulting in an increase in proliferation (Kissil et al., 2002). In contrast, Xenopus Pak1 acts to arrest cells at mitotic metaphase during embryogenesis (Rooney et al., 1996), and Xenopus Pak3 arrests the cell cycle and promotes neuron differentiation during neurogenesis (Souopgui et al., 2002), suggesting that in some contexts PAKs inhibit proliferation.
I found that, in *Dictyostelium*, the putative kinase PakD negatively regulates proliferation. At low cell densities, *pakD*<sup>−</sup> cells proliferate at the same rate as wild-type cells, but *pakD*<sup>−</sup> cells reach a higher maximum cell density than wild-type cells. PakD is required for the proliferation-inhibiting activity of both AprA and CfaD. Further, PakD is required for the chemorepellent effect of AprA, and *pakD*<sup>−</sup> cells show an increase in the size of filopodia, suggesting a role for PakD in the regulation of actin dynamics. Our data suggest that PakD is a regulator of proliferation and cell movement that functions downstream of AprA and CfaD.

**Materials and Methods**

To measure cell size by flow cytometry, cells were grown in shaking culture starting at 2 x 10<sup>5</sup> cells/ml and the average forward scatter of cells was measured daily using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

To image PakD-GFP localization by deconvolution microscopy, spots of *actin15::PakD-GFP* cells were grown in a 1.5 ml volume of HL5 in 2-well glass chamber slides (Nunc) overnight, and cells were subsequently fixed and stained with DAPI as described previously (Phillips et al., 2011). Cells were then imaged using an Olympus FV1000 microscope with a 100X 1.2 NA objective, and image z-stacks were generated with a slice separation of 0.2 microns. Z-stacks were then processed using Autodeblur deconvolution software (Bitplane software, Zurich, Switzerland). To stain cells with Alexa Fluor 594 Phalloidin (Invitrogen, Carlsbad, CA), cells were fixed as described above and then stained with phalloidin as previously described (Tang and
Gomer, 2008). To label the centrosome in cells expressing PakD-GFP, spots of actin15::PakD-GFP cells were grown in glass chamber slides overnight, and cells were then fixed for 30 minutes with 4% paraformaldehyde in PHEM buffer (30 mM Na-PIPES, 12.5 mM HEPES, 5mM EGTA, 1mM MgCl2, pH 6.9 (Graf, 2001)). Cells were washed three times in PBS and permeablized in PBS with 0.1% NP-40 for 10 minutes. Cells were then stained with anti-DdCP224 antibodies as previously described (Graf et al., 2000). Cells were then mounted in Vectashield mounting media with DAPI (Vector, Burlingame, CA) and imaged as described above. To image PakD- GFP in live, motile cells, spots of pakD^-/act15::PakD-GFP cells were grown in 2-well glass chamber slides (Nunc) overnight in FM media (Formedium, Norwich, UK). Cells were then imaged using a 100X objective by time-lapse microscopy. To image PakD-GFP with a marker for the Golgi apparatus, cells were grown overnight in low-fluorescence media (Bokko et al., 2007) in shaking culture and then were allowed to settle in borosilicate glass-bottom chamber slides (Nunc) for 15 minutes. Media and unadhered cells were removed by pipetting, and cells were then incubated for 1 hour in low-fluorescence media with 1 μg/ml wheat germ agglutinin conjugated to Alexa Fluor® 594 dye (Invitrogen). The stain was then pipetted off and replaced by low-fluorescence media, and cells were imaged as described above.

Results

PakD negatively regulates cell proliferation

Kinases of the p21-activated kinase (Pak) family are involved in signal transduction pathways regulating processes such as cell motility (Asano et al., 2009) and
proliferation (Rooney et al., 1996). PakD is a 190 kD PAK protein in *Dictyostelium* with putative kinase, diacylglycerol-binding, Cdc42/Rac interactive binding (CRIB), and calponin-homology (which functions in actin binding (Korenbaum and Rivero, 2002)) domains. Because AprA and PAKs affect both proliferation and motility, I examined whether PakD plays a role in AprA/CfaD signaling. Cells lacking AprA or CfaD show a faster doubling time than wild-type cells during logarithmic growth and reach a higher stationary density than wild-type cells (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). I found that pakD− cells showed a doubling time like wild-type cells during the logarithmic growth phase, but that pakD− cells proliferated to a significantly higher cell density than wild-type cells, and that this phenotype could be rescued by expression of a PakD-GFP transgene (Figure 32 and Table 11). These results indicate that PakD inhibits proliferation at high cell density and that pakD− cells exhibit some but not all of the phenotypes of aprA− and cfaD− cells.

As AprA and CfaD are extracellular signals that slow proliferation and increase in concentration at high cell density, one explanation for an increased proliferation phenotype is a lack of extracellular AprA or CfaD. To test this possibility, I examined the levels of AprA and CfaD in high-density conditioned media from pakD− cells by Western blot. pakD− and pakD−/act15::PakD-GFP cells showed extracellular accumulation of AprA and CfaD similar to or higher than that of wild type (Figure 33). These results strongly suggest that the high cell density of pakD− cells is not due to a lack of AprA or CfaD.
Figure 32: Cells lacking PakD proliferate to a higher density than wild-type cells. Cells were inoculated into axenic shaking culture at $1 \times 10^5$ cells/ml and counted daily. Values are mean ± SEM, n ≥ 3. The absence of error bars indicates that the SEM is smaller than the plot symbol.
Table 11. The effect of PakD on the doubling time and stationary density of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Doubling time, hours</th>
<th>Maximum observed cell density, $10^6$ cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>13.6 ± 0.3</td>
<td>24.3 ± 0.7</td>
</tr>
<tr>
<td>$pakD^-$</td>
<td>12.0 ± 0.6</td>
<td>43.6 ± 2.7***</td>
</tr>
<tr>
<td>$pakD^-$/actin15::pakD-GFP</td>
<td>13.7 ± 0.6</td>
<td>29.3 ± 1.3</td>
</tr>
</tbody>
</table>

Doubling times and stationary densities were calculated for the proliferation curves in Figure 1. Values are mean ± SEM from three independent experiments. “***” indicates that the difference between the value and the wild-type value is significant with $p < 0.001$ (one-way ANOVA, Tukey’s test). The difference between the maximum observed density of $pakD^-$ and $pakD^-$/pakD-GFP is significant ($p < 0.01$, Tukey’s test).
Figure 33: \( \text{pakD}^- \) cells accumulate extracellular \( \text{AprA} \) and \( \text{CfaD} \). Conditioned media from cells at \( 1 \times 10^7 \) cells/ml was collected and stained for AprA and CfaD by Western blots. The asterisk indicates a 27-kDa breakdown product of CfaD. Densitometry from 3 independent experiments showed that the intensity of this 27kDa band was not significantly different between genotypes, with bands from \( \text{pakD}^- \) and \( \text{pakD}^-/\text{PakD-GFP} \) genetic backgrounds showing 137 \( \pm \) 30% and 108 \( \pm \) 31% of the intensity of the band in the wild-type background, respectively. The non-specific band at 35 kDa in the AprA blot varied in intensity between experiments and between genotypes.
Recombinant AprA (rAprA) and CfaD (rCfaD) slow the proliferation of wild-type cells (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). To determine whether PakD plays a role in AprA and CfaD-mediated inhibition of proliferation, I examined the effects of rAprA and rCfaD on pakD− cells. As observed previously, wild-type cells showed reduced proliferation in response to rAprA and rCfaD (Figure 34). In contrast, pakD− cells showed no significant reduction in proliferation in the presence of either rAprA or rCfaD. rAprA and rCfaD slowed the proliferation of pakD−/act15::PakD-GFP cells, showing that the insensitivity of the pakD− cells is due specifically to the absence of the pakD gene. Together, these results indicate that PakD is a negative regulator of proliferation and that PakD is necessary for proliferation inhibition by AprA and CfaD.

pakD− cells show normal nuclei, mass and protein content per cell during logarithmic growth but are smaller than wild-type cells at stationary density

aprA− and cfaD− cells have more nuclei per cell than wild-type cells during vegetative growth, which may be due to an increased mitotic rate (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). I examined the nuclei content of log-phase pakD− cells by DAPI staining, and found that there was no significant difference as compared to wild type (Table 12). These results show that PakD does not affect the multinuclearity of cells, and suggest that AprA and CfaD affect cellular nuclei content in a manner independent of PakD.

Cell proliferation and cell growth (the accumulation of mass) can be regulated independently (Jorgensen and Tyers, 2004). AprA and CfaD regulate cell proliferation,
Figure 34: pakD<sup>−</sup> cells are insensitive to proliferation inhibition by AprA and CfaD.

Log-phase cells were collected, resuspended to 0.5 x 10<sup>6</sup> cells/ml, and either 300 ng/ml rAprA, 150 ng/ml rCfaD, or an equivalent volume of buffer was added to the cell culture. “*” indicates that the difference in values between the conditions are significant with p < 0.05, and “***” indicates p < 0.001 (One-way ANOVA, Tukey’s test). Values are mean ± SEM, n ≥ 3. For both rAprA and rCfaD, the values for pakD<sup>−</sup> cells are not significantly different from a value of zero (p > 0.05, paired t-test). For rAprA, but not for rCfaD, the differences between WT and pakD<sup>−</sup>/PakD-GFP are significant (p < 0.05, One-way ANOVA, Tukey’s test).
Table 12. The effect of PakD on the mass, protein, and nuclei content of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Per 10^7 cells</th>
<th>% cells with n nuclei</th>
<th>Nuclei/100 cells</th>
<th>Per 10^7 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td>Protein (mg)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Wild type</td>
<td>6.1 ± 0.3</td>
<td>0.41 ± 0.03</td>
<td>71 ± 2</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>pakD^-</td>
<td>5.3 ± 0.3</td>
<td>0.36 ± 0.02</td>
<td>73 ± 3</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>pakD^-/actin15::pakD-GFP</td>
<td>5.6 ± 0.6</td>
<td>0.43 ± 0.03</td>
<td>80 ± 5</td>
<td>18 ± 5</td>
</tr>
</tbody>
</table>

Mass and protein content were determined as described in the Materials and Methods. Values are the mean ± SEM from three or more independent experiments. Values for pakD^- or pakD^-/actin15::pakD-GFP were not significantly different from wild-type values for any parameter shown (p > 0.05, one-way ANOVA, Tukey’s test).
but not growth on a per nucleus basis (Bakthavatsalam et al., 2008; Brock and Gomer, 2005). To determine whether PakD affects cell mass or cell growth, I first measured the mass and protein content of cells. During exponential growth, wild-type cells showed mass and protein values like those seen previously (Table 12, Phillips and Gomer, 2010)). pakD− cells and pakD−/act15::PakD-GFP showed mass and protein content per cell and per nucleus that were not significantly different than wild-type values, indicating that PakD does not affect mass or protein content. I then estimated growth by dividing the mass and protein values by the measured doubling times during exponential growth to calculate the mass and protein accumulation per hour. Wild-type values for mass and protein accumulation were similar to those seen previously (Phillips and Gomer, 2010), and the values for mass and protein accumulation were not significantly different between wild-type, pakD−, and pakD−/act15::PakD-GFP strains on either a per cell or per nucleus basis (Table 13). These results indicate that PakD does not affect the mass or protein accumulation of cells undergoing exponential growth.

Wild-type cells increase in size at stationary density (Soll et al., 1976). As pakD− cells proliferate differently than wild type at high cell densities, I examined the effects of high cell density on pakD− cell size using flow cytometry. As assayed by forward scatter, wild-type cells showed a significant increase in cell size as cell density increased (Figure 35). In contrast, pakD− cells did not show a significant increase in size at high cell densities. Together, these results indicate that PakD does not affect mass and protein content at low cell density during exponential growth, but PakD is required for the increase in cell size at higher cell densities.
Table 13. The effect of PakD on the mass and protein accumulation of cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Per 10^7 cells per hour</th>
<th>Per 10^7 nuclei per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td>Protein (µg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.45 ± 0.03</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>pakD^-</td>
<td>0.44 ± 0.04</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>pakD^-/actin15::pakD-GFP</td>
<td>0.41 ± 0.06</td>
<td>31 ± 3</td>
</tr>
</tbody>
</table>

Mass and protein values from Table 2 were divided by the observed doubling time of the respective genotype. Doubling times were calculated as described in Materials and Methods. Values are the mean ± SEM from three or more independent experiments. Values for pakD^- or pakD^-/actin15::pakD-GFP were not significantly different from wild-type values for any parameter shown (p > 0.05, one-way ANOVA, Tukey’s test).
Figure 35: Wild-type but not pakD<sup>-</sup> cells increase in size at high cell densities.

Cells were inoculated into axenic shaking culture at 2 x 10^5 cells/ml and analyzed daily by flow cytometry to determine cell density and cell size as measured by forward scatter. Values are mean ± SEM, with n = 3.
PakD localizes to puncta within cells

I examined the subcellular localization of PakD by imaging vegetative cells expressing a PakD-GFP fusion protein. PakD-GFP localized to punctate structures within the cell (Figure 36), and there tended to be one punctate structure per cell, although occasionally more than one PakD spot per cell was observed. A similar localization of the endogenous PakD protein has been seen by immunofluorescence (Derrick Brazill, unpublished observations). When examining PakD-GFP in live, motile cells, a similar punctate structure was seen, and occasionally localization at the rear cortex of the moving cell was observed (Figure 36B), suggesting a role in cell movement. PakD-GFP did not co-localize with the nucleus (Figure 37A), with anti-DdCP224 staining, a marker for the centrosome (Figure 37A, (Graf et al., 2000)) or with wheat germ agglutinin staining, a marker for the Golgi apparatus (Figure 37B). No change in PakD-GFP localization was seen in cells at high density or in cells incubated with high-density conditioned media, suggesting that PakD localization is not altered in response to high AprA or CfaD levels. These results indicate that PakD localizes to a punctate structure that is not the nucleus, centrosome, or Golgi and that PakD may be polarized during cell movement.

PakD affects the ability of colonies to spread and the response of cells to the chemorepellant AprA

AprA is an autocrine chemorepellent in vegetative Dictyostelium cells (Phillips and Gomer, 2012) that may facilitate the spreading out of dense groups of cells. Consistent with this model, colonies of cells lacking AprA show a reduced ability to
Figure 36: PakD tends to localize to a single puncta or at the rear of motile cells.

(A) act15::PakD-GFP cells were grown in glass chamber slides overnight, then fixed and stained with DAPI (blue), imaged by fluorescence microscopy using a 100X objective, and processed using Autodeblur deconvolution software (Bitplane software, Zurich, Switzerland). PakD-GFP signal (green) is shown superimposed on a transmitted light image. Bar is 10 μm. (B) Live pakD−/act15::PakD-GFP cells grown as a colony in glass chamber slides were imaged by fluorescence microscopy. The imaged cell is moving upwards, as seen by time-lapse microscopy. The arrow indicates PakD-GFP at the rear of the cell. A single punctate structure is also visible. The cell border is shown with a dashed line. Bar is 10 μm.
Figure 37: PakD does not colocalize with the centrosome or Golgi. (A) PakD-GFP (green) does not colocalize with either the cell nucleus (blue) or with staining for an antibody against ddCP224 (Red), a marker for the centrosome. Bar is 5 μm. (B) In live cells, PakD-GFP (green) shows a different localization and morphology than wheat germ agglutinin staining (Red), a marker for the Golgi apparatus. Under these imaging conditions, cells tended to exhibit a rounded morphology. Bar is 5 μm.
spread on bacterial lawns (Phillips and Gomer, 2010) despite the fact that \textit{aprA}\textsuperscript{−} cells proliferate rapidly under these conditions (Brock and Gomer, 2005). To examine whether PakD may be involved in this process, I examined the size of \textit{pakD}\textsuperscript{−} colonies on bacterial lawns. Wild-type colonies showed a rate of expansion similar to what we observed previously (Phillips and Gomer, 2010), whereas \textit{pakD}\textsuperscript{−} cells showed a significantly reduced rate of expansion (Figure 38), suggesting that PakD functions in the expansion of colonies. I then tested whether \textit{pakD}\textsuperscript{−} cells, like wild type cells, show directed movement away from areas of high cell density (Phillips and Gomer, 2012) by tracking cell movement at the edge of a cell colony. Under these conditions, wild-type and \textit{pakD}\textsuperscript{−} cells showed similar speed, and \textit{pakD}\textsuperscript{−}\textit{act15::PakD-GFP} cells were significantly slower than wild-type cells (Table 14). These results indicate that the reduced expansion of \textit{pakD}\textsuperscript{−} cells is not due to reduced cell speed. However, \textit{pakD}\textsuperscript{−} cells had a significantly reduced chemotactic index in the direction away from the cell colony as compared to wild-type cells, and this phenotype was rescued by expression of PakD-GFP in \textit{pakD}\textsuperscript{−} cells (Table 14). Together, these data indicate that \textit{pakD}\textsuperscript{−} cells show reduced ability to spread from areas of high cell density, but that this is not due to a reduced cell speed.

Wild-type cells show directed movement away from a source of rAprA, indicating that AprA is a chemorepellant (Phillips and Gomer, 2012). To test whether PakD is necessary for the chemorepellant activity of rAprA, I examined whether \textit{pakD}\textsuperscript{−} cells show movement away from a rAprA source. Whereas wild-type cells showed a bias in movement away from a rAprA source, \textit{pakD}\textsuperscript{−} cells showed no significant bias in
Figure 38: *pakD*<sup>−</sup> cells show a reduced rate of colony expansion. Serial dilutions of cells were spread on SM/5 plates with bacteria, and the diameter of individual colonies was measured daily. Values are mean SEM, n = 3. The absence of error bars indicates that the SEM is smaller than the plot symbol.
Table 14. The effect of PakD on the movement of cells at the edge of colonies.

<table>
<thead>
<tr>
<th></th>
<th>Cell speed (µm/minute)</th>
<th>Forward migration index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.7 ± 0.6</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>pakD^-</td>
<td>6.3 ± 0.2</td>
<td>0.08 ± 0.03**</td>
</tr>
<tr>
<td>pakD^-/actin15::pakD-GFP</td>
<td>4.6 ± 0.2***</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

Colonies of cells were established on glass chamber slides in HL5 media and, following a one-hour incubation at room temperature, cells at the edge of the colony were filmed. Videos were used to track cell movement, and the tracking data was used to calculate the given values using the “Chemotaxis and migration tool” ImageJ plugin (Ibidi). At least 10 randomly selected cells from each of 3 independent experiments were tracked. A positive forward migration index indicates directed movement away from the colony. “**” indicates p <0.01 (t-test).
movement, and this phenotype could be rescued by PakD-GFP expression (Figure 39). These results indicate that PakD is necessary for rAprA chemorepellant activity.

I next tested whether pakD− cells showed aberrant proliferation on lawns of bacteria by spreading 1000 cells on rich media plates with bacteria and counting the number of Dictyostelium cells at different time points. At 24 hours, the number of wild-type cells had increased approximately 100-fold, consistent with previous findings (Figure 40, (Phillips and Gomer, 2010)). The number of pakD− cells was not significantly different from that of wild type, though there were significantly less pakD−/act15::PakD-GFP cells at this time point, suggesting that overexpression of PakD may slow proliferation on bacteria. However, at 72 hours, there were significantly less pakD− cells than wild-type cells, and this reduced cell number was partially rescued by expression of PakD-GFP (Figure 40). At this timepoint, individual Dictyostelium plaques were visible on the bacterial lawns, and wild-type and pakD−/act15::PakD-GFP plaques showed more spreading than pakD− plaques. Our data suggest that, initially, PakD is not required for normal proliferation, though overexpression of PakD may slow proliferation. However, at later time points, reduced spreading of pakD− colonies may result in reduced cell proliferation.

PAKs regulate actin dynamics (Kumar et al., 2009). To examine whether PakD is involved in the regulation of actin-based structures, I examined the morphology of live cells and of fixed cells stained with phalloidin, an actin-binding molecule. Live, randomly motile vegetative pakD− cells showed enlarged filopodia-like extensions at the
Figure 39: *pakD*-* cells are insensitive to the chemorepellant activity of AprA.

Log-phase cells on glass coverslips were placed in either HL5 media alone or in HL5 media with a gradient of rAprA using an Insall chamber. Cells were filmed, and the displacement of individual cells over 1 hour in the direction of the rAprA gradient was measured. Displacement to the right of the origin indicates movement away from the rAprA source. The average displacement of cells is plotted from 3 independent experiments, with the displacements for at least 10 randomly selected cells measured for each experiment. “**” indicates *p* < 0.01, and “***” *p* < 0.001 (t-test).
Figure 40: PakD affects the proliferation of cells on bacterial lawns. 1000 cells were spread with bacteria on SM/5 plates, and at the indicated times Dictyostelium cells were washed off the plate and counted. Values are mean ± SEM, with n = 3. "*" indicates that p < 0.05, and "***" indicates that p <0.01 (One-way ANOVA, Tukey’s test). At 72 hours, the difference between wild-type and pakD^-/ PakD-GFP is not significant.
cell periphery as compared to wild-type cells (Figure 41A). Similarly, fixed \( pako^- \) cells showed enlarged spiked f-actin structures at the periphery (Figure 41B). Expression of PakD-GFP in \( pako^- \) cells resulted in a reduction of these structures. These results suggest that PakD negatively regulates the formation of filopodia in vegetative cells.

Discussion

The mechanisms by which chalones act to slow proliferation and thus regulate tissue size are poorly understood. Our data indicate that the kinase PakD is a negative regulator of proliferation and is necessary for the activity of the chalones AprA and CfaD, indicating that PakD is involved in chalone signaling. Like \( apra^- \) and \( cfaD^- \) cells, \( pako^- \) cells accumulate mass and protein on a per nucleus basis during exponential growth at a rate like that of wild type, indicating that PakD regulates proliferation but does not significantly affect cell growth. However, \( pako^- \) cells differ in some ways from \( apra^- \) or \( cfaD^- \) cells. First, whereas \( apra^- \) and \( cfaD^- \) cells show rapid proliferation even at lower densities (Bakthavatsalam et al., 2008; Brock and Gomer, 2005), \( pako^- \) cells only show a difference in proliferation as compared to wild type at high densities. Second, \( apra^- \) and \( cfaD^- \) cells are multinucleate, whereas \( pako^- \) cells are not. Therefore, PakD likely mediates some but not all of the affects of AprA and CfaD. As \( pako^- \) cells proliferate like wild type at lower densities, some separate branch of the signal transduction pathway must mediate the effects of AprA and CfaD for these lower density cells. This branch may involve the transcription factor BzpN, which is necessary for AprA/CfaD signaling and affects proliferation primarily at lower cell densities (Phillips et al., 2011).
**Figure 41:** *pakD−* cells show enlarged filopodia. (A) Live vegetative cells in submerged culture were imaged using an inverted microscope with a 60X objective. Bar is 10 μm. (B) Cells were grown in chamber slides in HL5 media overnight and were then fixed and stained with Alexa Fluor 594 phalloidin. Expression of PakD-GFP in the shown *pakD−/act15::PakD-GFP* cell was confirmed by the presence of a punctate PakD-GFP signal (not shown). Bar is 10 μm.
Whereas wild-type cells increase in mass at high cell densities, I found that $pakD^-$ cells do not show this mass increase, but instead become smaller. As cells reach higher densities, it becomes more likely that starvation will occur, which initiates a process of aggregation and multicellular development in *Dictyostelium*. It may be the case that the density-dependent inhibition of proliferation mediated by PakD allows cells to accumulate mass and resources, better preparing them for the process of multicellular development.

I found that PakD-GFP shows a punctate subcellular localization that tends to localize in a single spot, and that this structure is not the centrosome or part of the nucleus or Golgi. This punctate structure is likely not an artifact of GFP fusion, as the native PakD protein shows a similar punctate localization (Derrick Brazill, unpublished observations). As *Dictyostelium* has lost cilia during evolution (Pazour, 2004), PakD-GFP is not associating with a primary cilium. PakD thus may be part of an uncharacterized subcellular structure. Alternatively, PakD may form endogenous, functioning self-aggregates, analogous to proteins such as CPEB (Si et al., 2003) or Orb2 (Majumdar et al., 2012).

PAKS are predominantly considered promoters and not inhibitors of cell growth and proliferation and are considered oncogenic in some circumstances (Molli et al., 2009). However, PAKs function to inhibit proliferation during *Xenopus* development (Rooney et al., 1996; Souopgui et al., 2002), though it was unclear if this function was conserved. Our data suggests that PAKs also show antiproliferative activity in
Dictyostelium, suggesting conservation of this function. It may therefore be useful to determine whether any human PAKs show a similar function, and whether PAK activity is affected by autocrine signaling. As PAKs are regulated by Rho/Rac/cdc42-type GTPases, it will be interesting in the future to test whether such proteins negatively regulate proliferation in vertebrates or in Dictyostelium, which has several Rho GTPase orthologs.

Apart from the proliferation-inhibiting activity of PakD, I also found that PakD is necessary for the chemorepellent activity of AprA, but does not affect the average speed of cells. Further, PakD is involved in the negative regulation of actin-based structures at the cell periphery. One appealing model for chemorepulsion consists of the recruitment of active PakD to subcellular areas of high AprA signaling due to an AprA gradient. This polarized PakD activity could potentially inhibit the development of actin-based structures in a polarized, AprA-dependent manner and thus inhibit movement up an AprA gradient. This model is supported by our observation of PakD-GFP at the rear of cells, but further studies are required to test this model more rigorously.

Much remains to be understood about how endogenous chemorepellants function in eukaryotic cells. I have shown that the protein AprA is an autocrine chemorepellant in the tractable model organism Dictyostelium, and that PAKs, which play a role in some chemorepulsive processes in vertebrates (Neufeld and Kessler, 2008), also are necessary for chemorepulsion in Dictyostelium. A better understanding of chemorepulsive processes could be useful for resolving hazardous immune system inflammation
(Vianello et al., 2005) of for preventing the dispersion and metastasis of tumors (Bagci et al., 2009). The conservation of PAK function between *Dictyostelium* and metazoans suggests that further study of AprA-mediated chemorepulsion could reveal important, uncharacterized regulators of chemorepulsive processes.

The fact that *pakD* cells show only some phenotypic similarity to *aprA* and *cfaD* cells indicates that AprA and CfaD signal through a branching pathway that regulates multiple processes. Based on phenotypic data, a model of AprA/CfaD signal transduction is shown in Figure 41. AprA, or perhaps an AprA/CfaD complex, binds to an unknown G protein-coupled receptor that associates with Gα8. Signaling through Gα8 facilitates spore development, inhibition of proliferation, and chemorepulsion. QkgA is downstream of Gα8 and facilitates proliferation inhibition and chemorepulsion, but not spore development. The transcription factor BzpN is activated by an unknown extracellular signal and regulates the expression of proliferation-inhibiting factors that act downstream of QkgA. A parallel pathway not regulated by BzpN involves proliferation-promoting elements that attenuate the proliferation-inhibiting activity of the pathway. PakD acts downstream of QkgA and mediates chemorepulsion as well as some aspects of proliferation inhibition. Currently, we do not know if any interactions among pathway components are direct (for instance, phosphorylation of PakD by QkgA). Future biochemical studies may be useful in revealing any such interactions. It is important to note that all characterized components of this pathway show sequence or structural similarity to human proteins. Thus, continued study may inform our understanding of proliferation control and chemorepulsion in humans, which would be valuable in the
Figure 42: A model for AprA/CfaD signal transduction. CfaD and/or AprA signal through an unknown G protein-coupled receptor. It is unclear whether AprA and CfaD activate the same or different receptors, or whether binding between the two proteins affects receptor interaction. AprA binding activates Gα8, which mediates proliferation inhibition, spore viability, and chemorepulsion. QkgA is downstream of Gα8 and regulates proliferation and chemorepulsion. BzpN is activated by an unknown factor present at high cell density and activates proliferation inhibiting factor(s) through regulation of transcription. A branch of the pathway independent of BzpN has proliferation-promoting activity that attenuates pathway activity. PakD mediates chemorepulsion by AprA and also inhibition of proliferation.
development of therapeutics for conditions in which regulating proliferation or cell movement would be beneficial.
References


