The ROCO Kinase QkgA Is Necessary for Proliferation Inhibition by Autocrine Signals in Dictyostelium discoideum

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AprA and CfaD are secreted proteins that function as autocrine signals to inhibit cell proliferation in Dictyostelium discoideum. Cells lacking AprA or CfaD proliferate rapidly, and adding AprA or CfaD to cells slows proliferation. Cells lacking the ROCO kinase QkgA proliferate rapidly, with a doubling time 83% of that of the wild type, and overexpression of a QkgA-green fluorescent protein (GFP) fusion protein slows cell proliferation. We found that qkgA− cells accumulate normal levels of extracellular AprA and CfaD. Exogenous AprA or CfaD does not slow the proliferation of cells lacking qkgA, and expression of QkgA-GFP in qkgA− cells rescues this insensitivity. Like cells lacking AprA or CfaD, cells lacking QkgA tend to be multinucleate, accumulate nuclei rapidly, and show a mass and protein accumulation per nucleus like those of the wild type, suggesting that QkgA negatively regulates proliferation but not growth. Despite their rapid proliferation, cells lacking AprA, CfaD, or QkgA expand as a colony on bacteria less rapidly than the wild type. Unlike AprA and CfaD, QkgA does not affect spore viability following multicellular development. Together, these results indicate that QkgA is necessary for proliferation inhibition by AprA and CfaD, that QkgA mediates some but not all of the effects of AprA and CfaD, and that QkgA may function downstream of these proteins in a signal transduction pathway regulating proliferation.

Physiological processes that define and maintain the sizes of tissues are poorly understood. Although a number of characterized gene products negatively regulate the sizes of tissues (21, 23), the mechanism by which the activities of such gene products are controlled is unclear. One potential mechanism for tissue size regulation consists of tissue-specific autocrine signals that inhibit proliferation in a concentration-dependent manner (18). Since the extracellular concentration of such factors increases as a function of cell density and/or cell number, the proliferation-inhibiting function of these factors can limit tissue size. Considerable evidence for such factors has been reported. For instance, full hepatectomy in one of two rats with conjoined circulatory systems stimulated proliferation in the intact liver of the conjoined rat, suggesting the existence of a systemic factor produced by the liver that inhibits the proliferation of hepatocytes (16). However, only a small number of factors with analogous functional roles, such as myostatin, which regulates skeletal muscle size (30), and Gdf11, which negatively regulates neurogenesis in the olfactory epithelium (38), have been identified. The mechanisms by which such signals inhibit proliferation are not well understood. As such autocrine signals may serve to limit tumor growth (14, 20), elucidation of the identities of such factors and their associated signal transduction pathways may yield novel cancer therapies.

We have identified two such autocrine proliferation-repressing signals in the social amoeba Dictyostelium discoideum, a genetically and biochemically tractable model organism. The proteins AprA and CfaD are secreted by Dictyostelium and inhibit the proliferation of Dictyostelium cells in a concentration-dependent manner (4, 12). Cells in which the genes encoding either AprA or CfaD have been disrupted by homologous recombination proliferate rapidly, and cells overexpressing AprA or CfaD proliferate slowly (4, 11). Adding recombinant AprA (rAprA) or recombinant CfaD (rCfaD) to cells slows proliferation, demonstrating that these proteins function as extracellular signals (4, 12). In addition to exhibiting rapid proliferation, aprA− and cfaD− cells exhibit a multinucleate phenotype, strongly suggesting that AprA and CfaD are negative regulators of mitosis (4, 11). aprA− cells are insensitive to the proliferation-inhibiting effects of CfaD (12), and cfaD− cells are insensitive to AprA (4), indicating the necessity of both genes for proliferation inhibition and suggesting a common proliferation-inhibiting mechanism. The G protein complex subunits Go8, Go9, and Gβ3 are necessary for proliferation inhibition by AprA, and the addition of recombinant AprA to purified cell membranes increases binding of GTP to wild-type and go9− cell membranes but not go8− or Gβ3− membranes, indicating that AprA activates a proliferation-inhibiting signal transduction pathway of which Go8 and Gβ3 are components (5). The signal transduction pathway downstream of Go8 and the associated mechanism of proliferation inhibition are unknown.

Although the selective forces that have maintained functional autocrine proliferation inhibitors in proliferating Dictyostelium cells are unclear, AprA and CfaD may provide an advantage during the multicellular portion of the Dictyostelium life cycle. Upon starvation, Dictyostelium cells secrete pulses of the chemoattractant cyclic AMP, leading to cells streaming toward aggregation centers (15, 27). This process causes the formation of multicellular groups regulated in size by a secreted protein complex that stimulates stream breakup (9, 10). These groups develop into multicellular fruiting body structures composed of a mass of stress-resistant spores supported...
by an approximately 1-mm-high stalk (24). While the stalk cells inevitably die in an act of apparent altruism (31), the presence of nutrients stimulates spore germination and a continuation of proliferation (13). Following development, aprA− and cfaD− cells form fewer viable spores than the wild type (4, 11), suggesting that AprA and CfaD increase the fitness of Dictyostelium during development.

Like aprA− and cfaD− cells, Dictyostelium cells lacking the ROCO family kinase QkgA have an abnormally rapid proliferation (1). The ROCO protein family is widely conserved and is defined by the presence of a Ras of complex protein (Roc) domain followed by a C terminus of Roc (Cor) domain, which mediates homodimerization (19). In eukaryotes, these domains are commonly followed C terminally by a kinase domain with similarity to the tyrosine kinase-like (TKL) group of kinases (3, 26, 29). In Dictyostelium, other ROCO proteins function in cyclic GMP signaling (8, 35) and cytokinesis (2), and a total of 11 predicted ROCO proteins are present in the genome, 10 of which, including QkgA, encode kinase domains predicted to be catalytically active (17). The human genome encodes two ROCO kinases, which are expressed in a wide range of tissues (25, 40). Little is known regarding the physiological functions of these proteins, although the ROCO protein LRRK2 is implicated in a dominantly inherited form of Parkinson's disease (40) and negatively regulates neurite growth in rat cortical cultures (28).

In this report, we show that, like aprA− and cfaD− cells, qkgA− cells proliferate to a higher cell density than the wild type and tend to be multinucleate. Additionally, we show that 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, qkgA− cells (1) were a kind gift from Jeff Williams. AX2 wild-type, qkgA− clone DBS0236839 (1), aprA− clone DBS0235509 (11), and cfaD− clone DBS0324444 (4) cells were grown in HL5 medium (Formedium Ltd., Norwich, England) as previously described (10). To generate cells expressing the QkgA C terminus-green fluorescent protein (GFP) fusion, cells were transformed with the extrachromosomal vector QkgA-PDM323 (36), a gift from Wouter van Egmond and Peter van Haastert, and selected in 15 μg/ml Geneticin. For each transformation, at least two clones were analyzed and had similar proliferative phenotypes. For proliferation curve experiments, all cells were grown in the transformation, at least two clones were analyzed and had similar proliferative phenotypes. For proliferation curve experiments, all cells were grown in the transformation, at least two clones were analyzed and had similar proliferative phenotypes.

#### Calculation of doubling times
To calculate the doubling time (tD) of cells during exponential growth, the following equation was used: 

\[ t_D = \frac{\ln(2) \cdot \ln(P_n/P_0)}{P_n} \]

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 1 × 10^5 cells/ml.

#### Fluorescence microscopy
DAPI (4′,6-diamidino-2-phenylindole) staining and imaging of nuclei were done as described previously (11). For imaging of cells expressing QkgA-GFP, cells growing in HL5 medium were washed and resuspended in low-fluorescence axenic medium (7), grown overnight in shaking culture, collected by centrifugation at 1500 × g, and resuspended in low-fluorescence medium at a volume equivalent to that of the starting volume. A drop of cells was placed on a glass slide and imaged with a Zeiss Axiosplan II microscope. For deconvolution imaging, cells growing in shaking culture in HL5 medium at densities of 1 × 10^7 to 4 × 10^7 cells/ml were allowed to settle on a glass coverslip for 10 min, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized in 0.2% Triton X-100 in PBS for 2 min, washed thrice in PBS for 5 min, and mounted in Vectashield mounting medium 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 (per liter, 10 g glucose, 10 g proteose peptone, 1 g yeast extract, 1 g MgSO₄·7H₂O, 1.9 g KH₂PO₄, 0.6 g K₂HPO₄·H₂O) and grown in 8-well glass slides. Cultures were used for experiments in which Dictyostelium cells were spaced approximately 40 μm apart and the ratio of bacteria to Dictyostelium cells was approximately 10:1. Cells were then imaged using an inverted microscope with a 10× objective, and cell movement was recorded using a Panasonic CCTV VW-BC200 camera. Distances between the approximate centroids of cells during 5-min intervals were measured and scaled to a stage micrometer to determine random motility values in micrometers. For colony edge imaging, 1-μl volumes of a suspension of Dictyostelium cells in HL5 medium were added to cells and dispersants that were grown overnight and subsequently imaged with an inverted microscope using a 20× objective.

#### 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 (1), and the resulting qkgA− cells were observed to proliferate rapidly. We 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 (4, 11), reached a significantly higher stationary density than wild-type cells in axenic culture (Fig. 1A and Table 1). The observed doubling time and stationary density of qkgA− cells were not significantly different from those of aprA− cells examined simultaneously (P > 0.05, 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, we transformed cells with a QkgA-GFP fusion construct (with GFP at the C terminus of QkgA) under the control of the actin15 promoter (36) and confirmed expression by fluorescence microscopy (Fig. 2). The fusion protein showed what appeared to be a cytosolic or vesicular localization, and no change in localization was observed at high density or in response to rAprA and rCfaD (Fig. 2B and data not shown). Expression of QkgA-GFP in wild-type cells resulted in slow proliferation and a low stationary density compared to those of the wild type (Fig. 1A 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), consistent with recently published data (36). These results support the hypothesis that QkgA is a negative regulator of proliferation.

FIG. 1. QkgA slows proliferation and lowers the stationary density of cells. (A) Log-phase cells were inoculated into HL5 medium at 1 × 10^6 cells/ml, and cell densities were measured daily. Values are means ± SEMs (n ≥ 3) for all conditions. WT, wild type. (B) One thousand cells were plated on SM/5 plates with K. aerogenes bacteria, and the total number of cells was determined daily. By 72 h, cells had begun to overgrow the bacteria. Values are mean ± SEMs (n = 4 for all conditions). (C) Cell numbers per plate at 24 h. The absence of error bars indicates that the error was smaller than the plot symbol. *, P < 0.05; **, P < 0.01 (repeated-measures one-way ANOVA, Tukey’s test). ns, not significant.

TABLE 1. Effect of QkgA on the doubling time and stationary density of cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Doubling time (h)</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 Materials and Methods. Values are means ± SEMs from five or more independent experiments. *, P < 0.05 (the difference between the value and the wild-type value is significant); **, P < 0.01 (one-way ANOVA, Tukey’s test).
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 levels in the wild type. To determine if low extracellular levels of AprA or CfaD might be responsible for the fast proliferation of **qkgA** cells, we 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 the wild-type levels (Fig. 3), 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 signals through QkgA to repress proliferation, we expected that **qkgA** cells would show some degree of insensitivity to AprA and/or CfaD. To test this prediction, we incubated proliferating cells with recombinant AprA (rAprA) or rCfaD and determined the percent decrease in cell density compared to that of a buffer control after a 16-hour incubation. Wild-type cells exhibited an approximately 15% decrease in cell density in response to rAprA or rCfaD (Fig. 4), which is on the order of what we have previously observed (4, 5, 12). 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 either rAprA/rCfaD or buffer was added showed no significant differences in cell density between the two conditions for **qkgA** cells, indicating that rAprA and rCfaD have no 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 (4, 11). To determine whether **qkgA** cells show this phenotype, we counted the number of nuclei. **qkgA** cells are multinucleate, whereas **aprA** and **cfaD** cells are not. These results suggest that QkgA is required for the multinucleate phenotype.

**FIG. 2.** Expression of QkgA-GFP in wild-type and **qkgA** cells. (A) Cells of the indicated genotype were grown in low-fluorescence axenic medium for 24 h and then washed once in medium and imaged by fluorescence microscopy using a 40× objective. Scale bar, 50 μm. DIC, differential interference contrast. (B) **qkgA**/act15::QkgA-GFP cells grown in HL5 medium were fixed in 4% paraformaldehyde and imaged with a 60× objective. The image was subsequently three-dimensionally deconvoluted. Scale bar, 1 μm.

**FIG. 3.** **qkgA** cells secrete AprA and CfaD. Conditioned medium from wild-type and **qkgA** cells at log phase (3 × 10^6 cells/ml) (A) or after log phase (12 × 10^6 cells/ml) (B) were assayed by Western blotting with anti-AprA antibodies (left) or anti-CfaD antibodies (right). Data are representative of three independent experiments. The asterisk indicates a breakdown product of CfaD. Numbers at the left indicate molecular masses in kDa.
nuclei per cell of DAPI-stained log-phase cells. \(qkgA^{-}\) cells were significantly less likely to have one nucleus per cell than the wild type and significantly more likely to have either two nuclei or three or more nuclei per cell than the wild type (Table 2). Expression of QkgA-GFP in \(qkgA^{-}\) cells rescued this multinucleate phenotype. Overexpression of QkgA-GFP in \(qkgA^{-}\) cells resulted in significantly fewer binucleate cells than wild-type cells. These results indicate that QkgA negatively regulates the number of nuclei per cell, perhaps by inhibiting mitosis. 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 (22). Although \(aprA^{-}\) and \(cfaD^{-}\) cells are more massive than wild-type cells, mass and protein are not accumulated in cells of these genotypes at a rate higher than that of the wild type on a per-nucleus basis, suggesting that \(qkgA^{-}\) 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 that of the wild type. When examined on a per-nucleus basis, the masses and protein accumulations 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 the wild type (4, 11). We examined the ability of \(qkgA^{-}\) cells to generate detergent-resistant spores by allowing equivalent numbers of wild-type and \(qkgA^{-}\) cells to develop, by collecting spores, and by plating dilutions of detergent-treated spores. The number of visible phase-dark, ovoid spores collected following development was 102\% \pm 4\% of the input cell number for the wild type and 117\% \pm 1\% for \(qkgA^{-}\) cells (mean \pm standard error of the mean [SEM], \(n = 3\)). Although the difference between the numbers of recovered spores from wild-type and \(qkgA^{-}\) cells was significant (\(P < 0.05\), \(t\) 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 3), suggesting that this difference was the result of the multinuclearity of \(qkgA^{-}\) cells. As judged by plaque number after cells were plated on bacteria, the number of viable spores after detergent treatment was 67\% \pm 5\% of the number of spores plated for wild-type cells and 68\% \pm 9\% for \(qkgA^{-}\) cells (mean \pm SEM, \(n = 3\)). Differences in detergent-resistant spore numbers between wild-type and \(qkgA^{-}\) cells were not significant (\(t\) test). Using an alternative spore viability assay (11), we also observed no sig-

![Inhibition by rAprA](image1.png)  ![Inhibition by rCfaD](image2.png)

**FIG. 4.** The expression of QkgA-GFP rescues the insensitivity of \(qkgA^{-}\) cells to rAprA (left) and rCfaD (right). Proliferating cells were incubated for 16 h with either 1,000 ng/ml rAprA, 600 ng/ml rCfaD, or an equivalent volume of buffer, and cell densities were then determined. The percent inhibition of cell density compared to that of a buffer control is shown. Values are means \pm SEMs (\(n \geq 4\) for all conditions). * \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\) (one-way ANOVA, Tukey’s test).

| TABLE 2. Effect of QkgA on the number of nuclei per cell* |
|---|---|---|
| Cell type | \(n = 0\) | \(n = 1\) | \(n = 2\) |
| Wild type | 78 \pm 2 | 20 \pm 1 | 2 \pm 1 |
| \(qkgA^{-}\) | 60 \pm 2* | 30 \pm 1** | 9 \pm 1*** |
| \(qkgA^{-}/\text{actin15}:\text{QkgA-GFP}\) | 81 \pm 2 | 18 \pm 2 | 1 \pm 1 |
| \(qkgA^{-}/\text{actin15}:\text{QkgA-GFP}\) | 85 \pm 1 | 14 \pm 1* | 1 \pm 1 |

*Log-phase cells were fixed and stained with DAPI, and the number of nuclei per cell for at least 200 cells per genotype was determined by fluorescence microscopy. Values are means \pm SEMs from three independent experiments. * \(P < 0.05\) (the difference between the value and the wild-type value is significant); ** \(P < 0.01\); *** \(P < 0.001\) (one-way ANOVA, Tukey’s test).
significant difference between wild-type spore viability and qkgA− spore viability (data not shown). These results suggest that QkgA does not affect the development of spores.

**Colonies of cells lacking AprA, CfaD, or QkgA expand slowly.** We 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 the wild type. To examine this apparent phenotype and to determine if qkgA− cells are similar, we plated serial dilutions of cells on bacterial lawns and measured the sizes of colonies. Under these conditions, the expansion of colonies of aprA− and cfaD− cells was less than that of the wild type (Fig. 5). Additionally, colonies of qkgA− cells showed a rate of expansion less than that of the wild type but similar to those 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.

As a reduced-colony-expansion phenotype could be due to defects in cell motility, we examined the random motility of cells cultured at low densities with bacteria on glass slides using video microscopy. During a 5-min interval, we observed an average displacement of wild-type, aprA−, cfaD−, and qkgA− cells of 7.6 ± 0.6 μm, 10.4 ± 1.1 μm, 8.6 ± 0.6 μm, and 10.5 ± 0.7 μm, respectively (means ± SEMs from three independent experiments with at least 10 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 that of the wild type and suggest that the reduced colony expansion observed in these mutants is not due to a defect in cell motility.

We 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 the 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 (Fig. 6). 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 (1). We found that qkgA− cells, like aprA− and cfaD− cells, have a high stationary density in shaking culture, multinuclearity and rapid accumulation of nuclei, a rate of growth per nucleus like that of the 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 cellular concentration of QkgA. We saw that qkgA− cells expressing QkgA-GFP proliferate like wild-type cells during log-phase growth, although qkgA−/act15:qkgA-GFP cells proliferate to a higher stationary density than the wild type. It may be the case, therefore, that native QkgA has increased activity at higher cell densities but that 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 ex-
pression may be reduced at high cell densities due to plasmid loss.

Like aprA<sup>−</sup> and cfaD<sup>−</sup> cells, qkgA<sup>−</sup> cells tend to be multinucleate. Although a cytokinesis defect could be responsible for this phenotype, this seems unlikely, as cells with a cytokinesis defect are expected to proliferate slowly rather than rapidly. We observed that QkgA inhibits the accumulation of nuclei in cells. 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<sup>−</sup> cells.

When estimating the accumulation of mass and protein per hour on a per-nucleus basis, we saw that qkgA<sup>−</sup> cells showed a higher mass and protein accumulation than the wild type, though these differences were not significant, suggesting that qkgA<sup>−</sup> cells accumulate mass and protein per nucleus at a rate like that of the wild type. The mass of wild-type cells increases as a function of cell density (33). As qkgA<sup>−</sup> cells proliferate rapidly but accumulate mass at a rate like that of the 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 show that qkgA<sup>−</sup> cells at stationary density do not decrease in number more rapidly than the wild type and that qkgA<sup>−</sup> cells do not show a defect in generating viable spores.

As aprA<sup>−</sup>, cfaD<sup>−</sup>, and qkgA<sup>−</sup> 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. Although aprA<sup>−</sup> and cfaD<sup>−</sup> cells yield fewer viable spores after development than the wild type, qkgA<sup>−</sup> 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, we saw that colonies of aprA<sup>−</sup>, cfaD<sup>−</sup>, and qkgA<sup>−</sup> 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

*FIG. 5. AprA, CfaD, and QkgA affect the expansion of colonies on bacterial lawns. Serial dilutions of log-phase cells in shaking culture (1 × 10<sup>6</sup> to 4 × 10<sup>6</sup> cells/ml) were mixed with bacteria and spread on SM/5 plates, and the average diameters of well-spaced colonies were determined daily. Values are means ± SEMs (n ≥ 3). The absence of error bars indicates that the error was smaller than the plot symbol. For the wild type, plates were overgrown after day 8, and colony boundaries could not be determined after this point.*

*FIG. 6. 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 *K. aerogenes* bacteria, and interfaces between bacteria and *Dictyostelium* cell populations were imaged. Scale bar, 100 μm.*
number at an optimal rate, and the cell population depends upon expansion to attain this quantity of nutrients. Thus, although aprA\textsuperscript{−}, cfaD\textsuperscript{−}, and qkgA\textsuperscript{−} cells may proliferate more rapidly than the 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 we saw that qkgA\textsuperscript{−} cells proliferate more than the wild type after a 24-hour period of growth in the presence of bacteria, it seems unlikely that qkgA cells are deficient in utilizing bacteria as an energy source. aprA\textsuperscript{−}, cfaD\textsuperscript{−}, and qkgA\textsuperscript{−} cells aggregate to form fruiting bodies and show random cell motility in the presence of bacteria, like 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 functions as a chemorepellent and thus aids 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\textsubscript{G}, which we have shown to be essential for AprA\textsuperscript{−} signaling (5), or the kinase YakA also proliferate rapidly in culture (5, 34) but show a reduced colony expansion phenotype (37, 39).

Currently, we cannot distinguish whether QkgA is actively involved in AprA/CfaD signaling (for instance, in a phosphocascade) 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\textsuperscript{−} cells proliferate rapidly suggests that QkgA functions specifically in negative regulation of proliferation as opposed to having 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 kinase for genes that mediate cell cycle progression identified a ROCO kinase family member as a potential regulator of cell cycle phase and centrosomal function (6). 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.

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