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A Retinoblastoma Orthologue Is Required for the Sensing of a Chalone in *Dictyostelium discoideum*

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Retinoblastoma-like proteins regulate cell differentiation and inhibit cell proliferation. The *Dictyostelium discoideum* retinoblastoma orthologue RblA affects the differentiation of cells during multicellular development, but it is unclear whether RblA has a significant effect on *Dictyostelium* cell proliferation, which is inhibited by the secreted proteins AprA and CfaD. We found that *rblA*$^-$ cells in shaking culture proliferate to a higher density, die faster after reaching stationary density, and, after starvation, have a lower spore viability than wild-type cells, possibly because in shaking culture, *rblA*$^-$ cells have both increased cytokinesis and lower extracellular accumulation of CfaD. However, *rblA*$^-$ cells have abnormally slow proliferation on bacterial lawns. Recombinant AprA inhibits the proliferation of wild-type cells but not that of *rblA*$^-$ cells, whereas CfaD inhibits the proliferation of both wild-type cells and *rblA*$^-$ cells. Similar to *aprA*$^-$ cells, *rblA*$^-$ cells have a normal mass and protein accumulation rate on a per-nucleus basis, indicating that RblA affects cell proliferation but not cell growth. AprA also functions as a chemorepellent, and RblA is required for proper AprA chemorepellent activity despite the fact that RblA does not affect cell speed. Together, our data indicate that an autocrine proliferation-inhibiting factor acts through RblA to regulate cell density in *Dictyostelium*, suggesting that such factors may signal through retinoblastoma-like proteins to control the sizes of structures such as developing organs or tumors.

Although a considerable amount is known about factors that promote cell proliferation, relatively little is known about factors that inhibit cell proliferation, especially factors secreted by the proliferating cells themselves that could function in a negative feedback loop to regulate the density of cells in a tissue or the size of a group of cells. A variety of observations in mammalian systems indicate that there exist such negative feedback factors, called chalones, but with the exception of a muscle size-regulating chalone called myostatin, very little is known about chalones or their signal transduction pathways (1–13).

The eukaryote *Dictyostelium discoideum* lives as proliferating haploid cells that eat bacteria on soil surfaces. When the cells in a local region overgrow their food supply and starve, they aggregate together and develop into ~2-mm-tall fruiting bodies composed of a thin column of stalk cells supporting a mass of spore cells; physical dispersion of the spores then allows the formation of new colonies. We found that during proliferation, *Dictyostelium* cells secrete the proteins AprA and CfaD, which slow proliferation and thus function as chalones (14, 15). Extracellular levels of AprA and CfaD increase as a function of cell density, and cells lacking either AprA or CfaD proliferate more rapidly than wild-type cells, are multinucleate, and reach a higher stationary density than wild-type cells (14, 15). The addition of either recombinant AprA (rAprA) or rCfaD to wild-type cells slows proliferation (14–16). Cells lacking AprA or CfaD accumulate mass on a per-nucleus basis at a rate similar to that of wild-type cells, indicating that AprA and CfaD regulate proliferation but not cell growth (14, 15). As cells tend to starve when they reach high cell densities, slowed proliferation due to AprA and CfaD combined with unchanged cell growth may provide cells with stored resources that aid in survival under conditions of starvation. This is supported by the observations that *aprA*$^-$ and *cfaD*$^-$ cells die more rapidly than wild-type cells after reaching stationary density and that starved *aprA*$^-$ and *cfaD*$^-$ cells produce fewer viable spores than do wild-type cells (14, 15). AprA also acts as a chemorepellent, which helps to disperse cells at the edge of a colony (17).

Retinoblastoma (RB) is a key regulatory protein of the cell cycle, and mutations in retinoblastoma can increase the chance of developing a specific hereditary ocular tumor also called retinoblastoma (18). The *RB1* gene was the first identified tumor suppressor (19), and this led to the multihit model of cancer development (20). RB regulates entry into S phase by binding E2F transcription factors to prevent the expression of genes needed for S phase (21). When a cell nears the G_1/S cell cycle checkpoint, cyclin-dependent kinases phosphorylate RB, allowing E2F to initiate transcription of S-phase genes (22). In mice, *Rb* is an essential gene, and homozygous *Rb* knockouts die before birth, whereas *Rb* heterozygotes are viable but tend to develop pituitary tumors (23, 24).

The *Dictyostelium* protein RblA is a Rb orthologue (25). Levels of *rblA* mRNA are low in proliferating cells and then increase dramatically during late development (25). This expression pattern is supported by transcriptome sequencing (RNA-seq) data, which show that RblA expression is low but present in cells growing on bacteria but is then upregulated during starvation (26). Surprisingly, although RblA does appear to repress the expression of both S-phase- and M-phase-specific genes (27), cells with a disruption of *rblA* are viable and show a normal proliferation rate,

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This article is dedicated to the memory of Harry MacWilliams.

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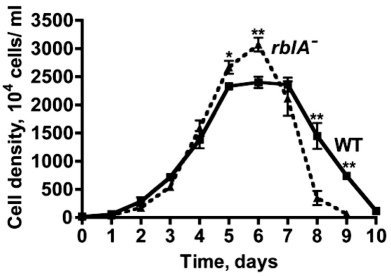

FIG 1 Cells lacking RblA proliferate to a higher cell density, and then die off faster, than wild-type cells. Vegetative wild-type (WT) and *rblA*$^-$ cells were diluted to 2×10^5 cells/ml in HL-5 medium, and the cell density was measured every day. Values are means ± SEM from three independent experiments. The absence of error bars indicates that the error was smaller than the plot symbol. * indicates a significant difference in the density of the two cell types, with a *P* value of <0.05, and ** indicates a *P* value of <0.01 (*t* test).

although cells overexpressing RblA proliferate very slowly. Proliferating *rblA*$^-$ cells tend to be smaller than wild-type cells (also indicating that RblA is expressed in vegetative cells), and *rblA*$^-$ cells undergo precocious but morphologically normal development (25). In this report, we show that RblA is necessary for AprA to inhibit proliferation and is required for proper AprA-induced chemorepulsion, indicating that in *Dictyostelium*, Rb is necessary for a pathway mediating the effects of a chalone.

MATERIALS AND METHODS

Cell culture; purification of recombinant proteins; and proliferation inhibition, chemotaxis, and spore viability assays. Wild-type Ax2 and *rblA*$^-$ cells (25) were cultured in HL-5 medium (Formedium Ltd., Norwich, England). Growth curves in liquid culture; production of rAprA and rCfaD; proliferation assays; nucleus counts; and calculation of doubling times, mass, and protein accumulation were done as previously described (14, 15). Proliferation on bacteria and colony sizes were measured according to methods described previously (28). Accumulation of extracellular AprA and CfaD was measured as described previously (29). Colony edge imaging was done as described previously (28). Chemotaxis of cells in response to rAprA was measured by using an Insall chamber (30) and a 0- to 2,000-ng/ml AprA gradient, as described previously (17). Cell speeds in the Insall chambers were measured according to methods described previously (31). Spore production and spore viability assays were done as described previously (28). To test the effect of CfaD levels on the maximum stationary density of *rblA*$^-$ cells, wild-type and *rblA*$^-$ cells were inoculated at 1×10^5 cells/ml, and cell density was monitored daily by hemocytometer counts. When cells had reached 1.82×10^7 to 2.15×10^7 cells/ml, the *rblA*$^-$ culture was split, and rCfaD at a final concentration of 84 ng/ml or an equivalent volume of 20 mM sodium phosphate buffer (pH 7.4) was added to the cells. The cell density was then measured daily by the use of a hemocytometer.

RESULTS

***rblA*$^-$ cells proliferate to an abnormally high cell density in liquid shaking culture.** To identify components of the *Dictyostelium* chalone signal transduction pathway, we have been examining genes that may affect proliferation. Previously reported growth curves of *rblA*$^-$ cells showed normal proliferation, but these growth curves went to only approximately 1.2×10^7 cells/ml (25). When we did a complete growth curve for *rblA*$^-$ cells, we also observed normal proliferation up to approximately 1.5×10^7 cells/ml, but at higher densities, the *rblA*$^-$ cells continued to proliferate, while, as previously observed (15), wild-type Ax2 cells, the parental line of *rblA*$^-$ cells, slowed and then stopped proliferation (Fig. 1). There was thus a significant increase in the stationary-phase density of *rblA*$^-$ cells compared to that of wild-type cells (Table 1). In addition, the *rblA*$^-$ cells then died off faster than did wild-type cells (Fig. 1). These results suggest that RblA slows and/or stops proliferation at high cell densities and promotes cell survival after cells have reached stationary phase in shaking cultures.

Before cells form visible colonies, *rblA*$^-$ cells show reduced proliferation on bacterial lawns. Although *aprA*$^-$ and *cfaD*$^-$ cells proliferate more rapidly than wild-type cells in liquid shaking culture, both *aprA*$^-$ and *cfaD*$^-$ cells, when plated onto bacterial lawns to form colonies, expand colonies less rapidly than do wild-type cells (28). To determine if *rblA*$^-$ cells also have this phenotype, dilutions of wild-type and *rblA*$^-$ cells were plated onto bacteria, and the average colony diameter was measured daily. *rblA*$^-$ cells formed significantly smaller colonies than those formed by wild-type cells (Fig. 2A). Interestingly, the colony diameters appeared to increase at roughly the same rate, with *rblA*$^-$ cells having a delay in colony formation. To more directly examine the proliferation of *rblA*$^-$ cells on bacteria, cells were mixed with bacteria and plated onto nutrient agar, and the number of *Dictyostelium* cells was counted daily before the cells had time to clear the bacterial lawns. At 48 and 72 h, the numbers of *rblA*$^-$ cells were significantly smaller than the numbers of wild-type cells (Fig. 2B). A possible explanation for these results is that when there are large numbers of bacteria and relatively few *Dictyostelium* cells, *rblA*$^-$ cells proliferate more slowly than wild-type cells (Fig. 2B), but when a colony forms and a high-density front of *Dictyostelium* cells moves across a lawn of bacteria, *rblA*$^-$ cells can proliferate and move at roughly the same rate as wild-type cells (Fig. 2A).

***rblA*$^-$ cells show reduced accumulation of extracellular CfaD but normal accumulation of AprA.** One possible reason why *rblA*$^-$ cells proliferate to a higher cell density than wild-type cells in liquid shaking culture is that they might accumulate less of the AprA and/or CfaD proliferation-inhibiting factors (14, 15). To test this possibility, we collected conditioned medium from proliferating cells and used Western blots, stained with affinity-purified anti-AprA and anti-CfaD antibodies, to examine AprA and CfaD levels. As shown in Fig. 3A, we did not observe any significant difference in the amounts of extracellular AprA in cultures of wild-type and *rblA*$^-$ cells, but we did observe that *rblA*$^-$ cells accumulated less extracellular CfaD. Densitometry of the 62-kDa CfaD band showed that when cells were at density of 5×10^6 cells/ml, *rblA*$^-$ cells accumulated 35% ± 9% (mean ± standard error of the mean [SEM]; $n = 3$) of the wild-type level of CfaD, which, at this cell density, is ~8 ng/ml (15). In addition, compared to wild-type cells, levels of the 27-kDa CfaD breakdown band (15) (Fig. 3A) were consistently low in conditioned medium from *rblA*$^-$ cells. These results suggested that one factor that may con-

TABLE 1 Effect of RblA on doubling time and stationary density of cells[a]

Cell type	Mean doubling time (h) ± SEM	Mean max observed cell density (10^6 cells/ml) ± SEM
Wild type	13.3 ± 1.0	24.0 ± 1.0
rblA$^-$	12.7 ± 0.6	30.7 ± 1.2**

[a] Values are means ± SEM ($n = 3$). ** indicates that the difference between the value and the wild-type value is significant, with a *P* value of <0.01 (*t* test).

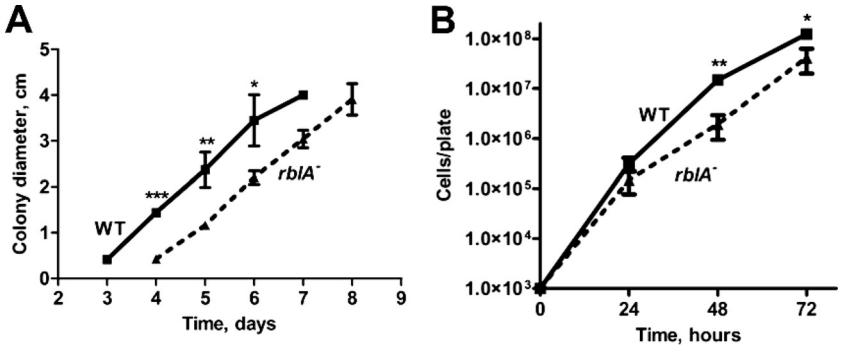

FIG 2 *rblA*$^-$ cells show reduced proliferation on bacterial lawns. (A) Dilutions of log-phase cells were mixed with bacteria and spread onto SM/5 plates. Well-spaced colonies were imaged daily, and the average colony diameter was measured. Before day 3 for wild-type cells and day 4 for *rblA*$^-$ cells, no clearing of the bacterial lawn was observed. (B) A total of 10^3 cells were plated onto bacterial lawns, and the number of *Dictyostelium* cells was counted daily. For panels A and B, values are means ± SEM ($n = 7$); the absence of error bars indicates that the error was smaller than the plot symbol. * indicates a P value of <0.05, ** indicates a P value of <0.01, and *** indicates a P value of <0.001 (t test).

tribute to the abnormally high stationary-phase cell density of *rblA*$^-$ cells is their abnormally low level of extracellular CfaD. To test this possibility, we grew *rblA*$^-$ cells to a cell density approaching stationary density and then split the culture and added either 84 ng/ml of rCfaD, a concentration that is present in high-density wild-type cultures (15), or an equivalent volume of buffer. We observed that *rblA*$^-$ cells proliferated to a higher density than did wild-type cells, and the addition of rCfaD did not significantly reduce cell density (Fig. 3B). These results suggest that the high cell density reached by *rblA*$^-$ cells is not due specifically to reduced levels of extracellular CfaD.

***rblA*$^-$ cells show a more complete extent of cytokinesis than do wild-type cells.** Cells lacking AprA, CfaD, and the AprA/CfaD signal transduction components Gα8, Gβ, QkgA, BzpN, and CnrN tend to be multinucleate (14, 15, 28, 29, 31, 32). We tested whether *rblA*$^-$ cells tend to be multinucleate by counting the number of nuclei per cell in proliferating cell populations. Counts of the numbers of nuclei per cell for wild-type cells were similar to what we have observed previously (Table 2) (14, 15). Compared to wild-type cells, there was a significant increase in the *rblA*$^-$ population of mononucleate cells and significant decreases in the percentages of cells with two and three or more nuclei (Table 2). The observation that the loss of RblA results in fewer multinucleate cells thus suggests that RblA inhibits cytokinesis.

***rblA*$^-$ cells show normal cell growth.** Cell growth (the accumulation of mass per cell or per nucleus) and cell proliferation can be regulated independently (33). Although *aprA*$^-$ and *cfaD*$^-$ cells tend to be more multinucleate than wild-type cells, *aprA*$^-$ and *cfaD*$^-$ cells show an accumulation of mass per nucleus like that of the wild type, suggesting that AprA and CfaD regulate proliferation but not growth (14, 15). To determine the effect of RblA on growth, we measured the mass and protein content of log-phase cells and calculated the accumulation of mass and protein per cell and per nucleus (i.e., the cellular mass or protein level divided by the number of nuclei per cell), using the measured doubling times (Table 1) and nucleus counts (Table 2). In agreement with previous observations that *rblA*$^-$ cells tend to be smaller than wild-type cells (25), we observed that *rblA*$^-$ cells tended to have less protein and fewer nuclei per cell than wild-type cells (Table 3), although values for mass and protein content were similar between wild-type and *rblA*$^-$ cells when judged on a per-nucleus basis. The accumulation of mass, nuclei, and protein per cell per hour or per nucleus per hour was not statistically different between *rblA*$^-$ and wild-type cells (Table 4). These results suggest that RblA does not affect cell growth.

***rblA*$^-$ cells show insensitivity to the proliferation-inhibiting effect of AprA.** Proliferation to an abnormally high cell density, and then a faster cell death than for wild-type cells after cells reach stationary phase, is characteristic of *aprA*$^-$ and *cfaD*$^-$ cells as well as cells lacking the AprA and CfaD signal transduction compo-

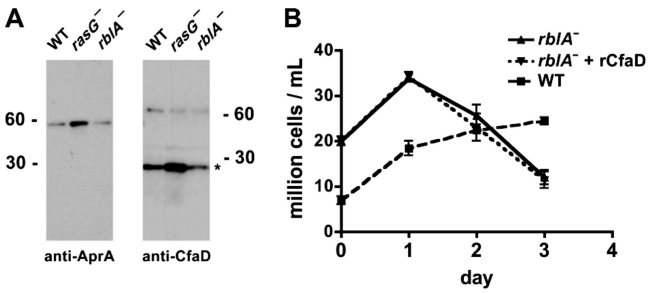

FIG 3 Cells lacking RblA accumulate less extracellular CfaD than do wild-type cells. (A) Proliferating cells were grown to 5×10^6 cells/ml, as described in the legend of Fig. 1, and the conditioned medium was collected. Western blots of the conditioned media were stained for AprA (left) or CfaD (right). This work was done as part of an experiment measuring extracellular AprA and CfaD levels in a variety of cell types; hence, extracellular levels of AprA and CfaD in *rasG*$^-$ cells are also shown. Molecular masses (in kDa) are indicated at left and right. Data are representative of 3 independent experiments. (B) A total of 84 ng/ml rCfaD or an equivalent volume of buffer was added at day 0 to *rblA*$^-$ cells approaching stationary density, and cell density was measured daily. Values are means ± SEM from three independent experiments. The absence of error bars indicates that the error was smaller than the plot symbol.

TABLE 2 Effect of RblA on the number of nuclei per cell[a]

Cell type	Mean % of cells with no. of nuclei ± SEM		
	1	2	3+
Wild type	72.9 ± 1.5	24.0 ± 1.2	3.1 ± 0.3
rblA$^-$	85.0 ± 1.3***	13.4 ± 0.8***	1.5 ± 0.5*

[a] Fluorescence microscopy was used to count the number of nuclei per cell (at least 800 cells under each condition) for log-phase cells stained with DAPI (4′,6-diamidino-2-phenylindole). Values are means ± SEM ($n = 3$). * indicates that the value is significantly different compared to the wild-type value, with a P value of <0.05, and *** indicates a P value of <0.005 (t test).

TABLE 3 Effect of RblA on mass and protein and nucleus contents of cells[a]

Cell type	Mean mass (mg)/10^7 cells ± SEM	Mean protein concn (mg)/10^7 cells ± SEM	Mean no. of nuclei/ 100 cells ± SEM	Mean mass (mg)/10^7 nuclei ± SEM	Mean protein concn (mg)/10^7 nuclei ± SEM
Wild type	11.6 ± 0.4	0.39 ± 0.01	132 ± 2	8.8 ± 0.3	0.30 ± 0.01
rblA$^-$	10.6 ± 0.3	0.33 ± 0.01*	117 ± 2**	9.0 ± 0.3	0.29 ± 0.01

[a] Mass and protein content were determined as described in Materials and Methods, and nucleus counts were obtained from the data summarized in Table 2. Mass and protein per 10^7 nuclei are the mass and protein values per 10^7 cells divided by the nucleus/cell number (the number in the third data column, divided by 100). Values are means ± SEM from three or more independent experiments. * indicates that the difference between the value and the wild-type value is significant, with a *P* value of <0.05, and ** indicates a *P* value of <0.01 (*t* test).

nents Gα8, Gβ, and CnrN (14, 15, 29, 31). Since *rblA*$^-$ cells have this phenotype, we thus determined whether *rblA*$^-$ cells have an abnormal response to AprA or CfaD. We used low-density log-phase cells in our assays, as *Dictyostelium* cells grow abnormally slowly at densities lower than this (34), and at higher densities, high levels of accumulated endogenous AprA and CfaD can interfere with the assay. We measured the effect of rAprA and rCfaD at 18 h, as we have observed that culturing of cells with exogenous recombinant proteins past 24 h leads to degradation and/or ingestion of the recombinant proteins (our unpublished data). As previously observed (15, 16), rAprA and rCfaD inhibited the proliferation of wild-type cells (Fig. 4). We found that rAprA was not able to suppress the proliferation of *rblA*$^-$ cells (Fig. 4A). Although it appears that rAprA slightly increased the proliferation of *rblA*$^-$ cells, the effect was not statistically significant (*t* test). The *rblA*$^-$ cells showed a normal response to rCfaD (Fig. 4B). These results indicate that RblA is required for the proliferation-inhibiting effect of AprA but not of CfaD. As we saw that the proliferation of high-density *rblA*$^-$ cells was not significantly inhibited by exogenous rCfaD (Fig. 3B), our results indicate that either extracellular CfaD levels that endogenously accumulate at high densities in *rblA*$^-$ cells provide the strongest proliferation-inhibiting response possible by CfaD (at levels above 80 ng/ml, further addition of rCfaD does not cause any additional inhibition of proliferation for wild-type cells [15]) or high-density cells that are no longer growing exponentially do not significantly respond to CfaD.

***rblA*$^-$ cells have reduced sensitivity to the chemorepellent effect of AprA.** High levels of extracellular AprA in the vicinity of a colony of cells, and lower levels far away from the colony, cause an AprA gradient that directs the chemotaxis of cells away from the colony (17). Since RblA is required for the effect of AprA on cell proliferation (Fig. 4), we determined if RblA might also be required for the chemorepellent effect of AprA by first examining the edges of colonies of *rblA*$^-$ cells. As previously observed (17), 24 h after plating of a small spot of cells onto a glass slide in HL-5 medium, colonies of wild-type cells showed cells scattered away from the colony edge (Fig. 5). At 24 h, this effect was not observed in colonies of *rblA*$^-$ cells, causing the colonies to form a relatively smooth edge (Fig. 5). Similar results were also observed at 48 h after plating (data not shown). These results suggest that RblA affects the behavior of cells at the edge of a colony.

To directly determine if RblA is involved in AprA gradient-induced chemorepulsion, we placed Ax2 and *rblA*$^-$ cells in a defined rAprA gradient. As previously observed (17), wild-type cells showed chemorepulsion away from rAprA (Fig. 6). *rblA*$^-$ cells also showed chemorepulsion away from rAprA, but the chemorepulsive effect was significantly reduced compared to that of wild-type cells. This reduced chemorepulsion could be due to either a reduced ability of *rblA*$^-$ cells to move on the glass surface used for the chemorepulsion assays or a defect in the ability to sense and respond to an AprA gradient. To distinguish between these possibilities, we examined the speed of wild-type and *rblA*$^-$ cells in the presence or absence of a rAprA gradient (Fig. 7). Cell speeds were similar to what we have previously observed (17), and there were no significant differences in the speeds of wild-type or *rblA*$^-$ cells in the presence or absence of a rAprA gradient. Together, these results suggest that RblA does not affect the ability of cells to move on a glass surface and that RblA is required for proper AprA-induced chemorepulsion.

***rblA*$^-$ cells have normal spore formation but reduced spore viability.** A drawback to the loss of AprA is a reduced number of spores after development, and reduced spore viability, if cells starve and form fruiting bodies (14). Since RblA appears to mediate some downstream effects of AprA, we determined whether RblA affects spore numbers and/or viability. After starvation and development, wild-type cells formed a similar number of spores, and these spores had a similar viability (as measured by resistance to detergent treatment) to what we have previously observed (14). Under similar conditions, *rblA*$^-$ cells formed a similar number of spores compared to the wild type (Fig. 8A). However, *rblA*$^-$ spores had an abnormally low viability (Fig. 8B). These results suggest that RblA is not required for spore formation but is required for normal levels of spore viability.

DISCUSSION

Retinoblastoma-like proteins show a broad conservation of two functions: inhibitory regulation of the cell cycle and regulation of cell differentiation (35, 36). Intriguingly, an analysis of the *Dictyostelium* RblA protein suggested that under conditions of physio-

TABLE 4 Effect of RblA on mass and protein accumulation of cells[a]

Cell type	Mean mass (mg)/10^7 cells/h ± SEM	Mean protein concn (μg)/10^7 cells/h ± SEM	Mean no. of nuclei (10^{-5}) ± SEM	Mean mass (mg)/10^7 nuclei/h ± SEM	Mean protein concn (μg)/10^7 nuclei/h ± SEM
Wild type	0.87 ± 0.07	29 ± 2	9.9 ± 0.7	0.66 ± 0.05	22 ± 2
rblA$^-$	0.83 ± 0.05	26 ± 1	9.2 ± 0.5	0.71 ± 0.04	22 ± 1

[a] Mass and protein 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 means ± SEM from three or more independent experiments.

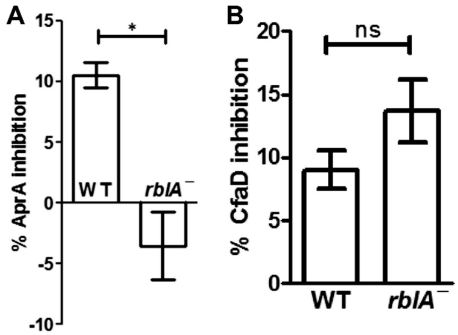

FIG 4 AprA requires RblA to slow proliferation. (A) The addition of 300 ng/ml rAprA slowed the proliferation of wild-type cells but not of *rblA*$^-$ cells (*, $P < 0.05$ [*t* test]). (B) The addition of 125 ng/ml rCfaD slowed the proliferation of both wild-type and *rblA*$^-$ cells (ns indicates that the value was not significant by *t* test). Values are means ± SEM from at least three independent experiments.

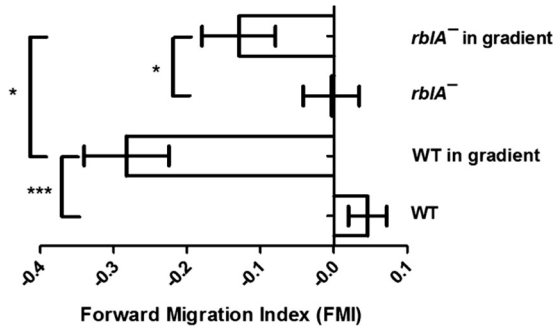

FIG 6 RblA is required for a proper response to the chemorepellent AprA. Cell movement in the presence or absence of an AprA gradient in an Insall chamber was observed by video microscopy. The movement of individual cells was tracked for an hour for four independent experiments, with at least 9 cells per condition being examined for each experiment. The forward migration index for each cell was then calculated. Negative values indicate a movement away from the source of rAprA. Values are means ± SEM of the averages of all measured forward migration index values under each condition. * indicates a *P* value of < 0.05 (*t* test). WT, wild type.

logical protein levels, RblA affects cell differentiation but does not have a significant inhibitory effect on the cell cycle (25). However, our study of RblA in the context of cell density-dependent regulation of proliferation by AprA indicates that RblA does in fact play a role in regulating cell proliferation. In shaking axenic culture and during proliferation on a bacterial lawn, *rblA*$^-$ cells proliferated at a rate equal to or lower than that of wild-type cells at lower cell densities, but *rblA*$^-$ cells reached a higher maximum cell density in shaking culture. These results implicate RblA as an inhibitor of proliferation of cells at very high densities. Our findings are consistent with a report of broad repression of cell cycle genes by RblA in *Dictyostelium* (27).

AprA and CfaD are autocrine signals that act as a readout of cell density and function to inhibit proliferation in a concentration-dependent manner (14, 15). We found that, in contrast to wild-type cells, the proliferation of *rblA*$^-$ cells is not inhibited by AprA, indicating that RblA acts downstream of a cell density-reporting signal that inhibits proliferation. These results are congruent with mammalian studies, which indicate that myostatin, a proliferation-inhibiting, cell density-dependent signal that acts on myoblasts, increases the accumulation of the active, proliferation-inhibiting form of Rb (37). However, we found that *rblA*$^-$ cells are sensitive to the proliferation-inhibiting activity of CfaD. The RblA mutant is the first mutant that we have analyzed that shows insensitivity to one but not the other of these two signals. Thus, the signaling pathways of AprA and CfaD diverge to some degree, as RblA is required for the activity of AprA but not of CfaD.

AprA acts as an autocrine chemorepellent that facilitates the spreading of groups of cells (17). We found that *rblA*$^-$ cells are able to sense and respond to an AprA gradient but less efficiently than wild-type cells. Although we found that *rblA*$^-$ cells and wild-type cells move at similar speeds and secrete similar levels of AprA, the edges of colonies of *rblA*$^-$ cells appear less dispersed than those of wild-type cell colonies, suggesting that the reduced chemorepulsion has significant physiological effects. To our knowledge, this is the first report of Rb involvement in a chemotactic process. Due to its known role as a regulator of gene expression, we speculate that RblA regulates the expression of genes involved in sensing or responding to a gradient of AprA as opposed to being directly involved in these processes.

Our data indicate that RblA mediates some but not all of the effects of AprA. Even during the logarithmic phase of growth, *aprA*$^-$ cells proliferate more rapidly than wild-type cells (14), whereas *rblA*$^-$ cell proliferation differs from that of the wild type only at very high densities. Furthermore, *aprA*$^-$ cells tend to be multinucleate, whereas *rblA*$^-$ cells do not show this phenotype. Finally, *aprA*$^-$ but not *rblA*$^-$ cells show a reduced number of

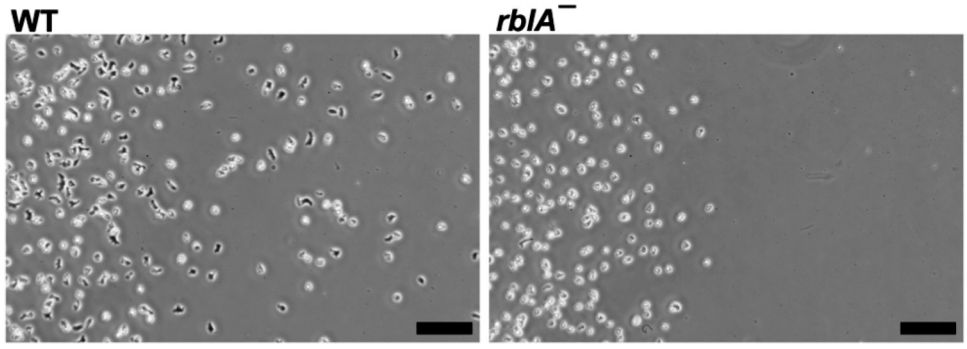

FIG 5 RblA is required for normal levels of cell dispersal at the edge of a colony. Wild-type (WT) or *rblA*$^-$ cells were inoculated onto a spot in the well of a 2-well slide chamber, and after 15 min to allow cell adhesion, HL-5 medium was added to the chamber. Images show colony edges 24 h after colony plating and are representative of at least 6 independent experiments. Bars are 100 μm.

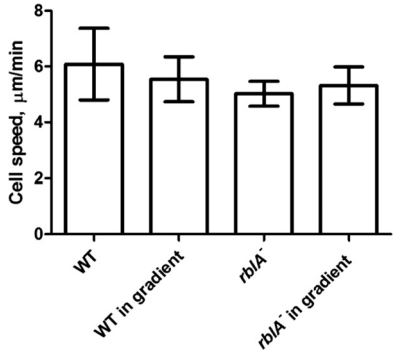

FIG 7 RblA does not affect cell speed on a glass surface. After measuring the position of each cell every 26 s for 1 h by video microscopy (Fig. 6), the speeds of cells along their movement tracks were measured. The average speed of all observed cells was then calculated. Values are means ± SEM of the averages from four independent experiments.

spores formed during development compared to the numbers formed by wild-type cells. These results suggest that AprA regulates multiple processes through branching signal transduction pathways and that RblA is located in a downstream position in the pathway, separated from the branches that regulate spore formation, multinuclearity, and some aspects of proliferation inhibition.

We found that on bacterial lawns, *rblA*$^-$ cells proliferate slowly and show a delay in the formation of visible colonies compared to wild-type cells. To date, no other mutants (*aprA*$^-$, *cfaD*$^-$, *cnrN*$^-$, *qkgA*$^-$, or *bzpN*$^-$) that we have screened within the chalone pathways have this phenotype (14, 15, 28, 29, 31, 32). A possible explanation for this is that although RblA mostly regulates the expression of cell cycle genes, it also regulates the expression of some genes that may be involved in the ingestion and/or digestion of bacteria (27). It is thus possible that the loss of RblA somewhat reduces the ability of *Dictyostelium* cells to proliferate on bacteria but, until cells reach very high densities, does not significantly affect the ability to proliferate in liquid shaking culture.

rblA$^-$ cells show accelerated development compared to wild-type cells, and this acceleration is most evident in the aggregative stage: wild-type cells aggregate in 10 to 12 h, whereas *rblA*$^-$ cells aggregate in 5 to 6 h (25). An interesting possibility is that AprA is also involved in developmental timing. As AprA acts as a chemorepellent, one potential mode of action would be that the chemorepulsive activity of AprA somewhat counteracts the chemoattractant cyclic AMP (cAMP), thus acting to somewhat delay aggregation. We have previously shown that AprA does not show significant chemorepulsive activity on cells starved for 5 h (17), although chemorepulsive activity at other time points might affect aggregation. Future studies may reveal whether AprA, like RblA, affects developmental timing.

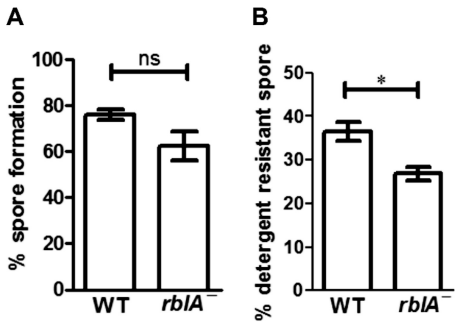

FIG 8 RblA is required for normal levels of spore viability. (A) Cells were starved, counted, and plated for development of fruiting bodies, and the number of spores was then counted to determine the percentage of the initial number of cells that formed spores. (B) A fixed number of spores was treated with detergent, diluted, and plated with bacteria, and the number of *Dictyostelium* colonies that developed from the germinating spores was then counted. The percentage of spores that formed viable colonies was then calculated. Values in panels A and B are means ± SEM of at least three independent experiments. * indicates a *P* value of <0.05, and ns indicates that the value was not significant (*t* test).

Together, our data indicate that RblA acts to inhibit cell proliferation at high densities and is required for the proliferation-inhibiting activity and proper chemorepellent activity of the chalone AprA. Currently, we cannot distinguish whether RblA is merely permissive for AprA signaling (for example, RblA may be required for the expression of an AprA signaling component) or an active, signal-transducing component of the pathway. One attractive model is that the AprA pathway might stimulate the activity of a protein phosphatase that acts on RblA. Sufficient pathway activity would keep RblA in a hypophosphorylated, active state, thus preventing progression of the cell cycle. Future studies may reveal whether such a phosphatase is acting in this system. Irrespective of the mechanism, the contribution of RblA to a pathway that senses an autocrine signal (AprA) that functions in a negative feedback loop to inhibit cell proliferation suggests the intriguing possibility that in other systems, autocrine signals may similarly signal through pathways regulated by retinoblastoma-like proteins to inhibit cell proliferation and, thus, tissue size.

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