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A secreted factor represses cell proliferation in *Dictyostelium*

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Summary

Many cells appear to secrete factors called chalones that limit their proliferation, but in most cases the factors have not been identified. We found that growing *Dictyostelium* cells secrete a 60 kDa protein called AprA for autocrine proliferation repressor. AprA has similarity to putative bacterial proteins of unknown function. Compared with wild-type cells, *aprA*-null cells proliferate faster, while AprA overexpressing cells proliferate slower. Growing wild-type cells secrete a factor that inhibits the proliferation of wild-type and *aprA*$^-$ cells; this activity is not secreted by *aprA*$^-$ cells. AprA purified by immunoprecipitation also slows the proliferation of wild-type and *aprA*$^-$ cells. Compared with wild type, there is a higher percentage of multinucleate cells in the *aprA*$^-$ population, and when starved, *aprA*$^-$ cells form abnormal structures that contain fewer spores. AprA may thus decrease the number of multinucleate cells and increase spore production. Together, the data suggest that AprA functions as part of a *Dictyostelium* chalone.

Keywords

Chalone; Proliferation; Growth; Size regulation

Introduction

A variety of observations have suggested the existence of autocrine factors called chalones that negatively regulate the growth and/or proliferation of the secreting cells (Gamer et al., 2003; Gomer, 2001). For example, myostatin is a polypeptide belonging to the platelet-derived growth factor superfamily that is made by, and secreted from, myoblasts. As the percent of the body occupied by muscle increases, the serum concentration of myostatin increases (Lee and McPherron, 1999). Myostatin negatively regulates myoblast proliferation, and this negative feedback maintains the amount of muscle in the body (Thomas et al., 2000). Mutations in myostatin result in abnormally large muscles in mice and cattle (McPherron and Lee, 1997; McPherron et al., 1997). Muscle size is thus regulated by a feedback loop involving a secreted factor that is used to sense the number of cells secreting the factor, with the factor inhibiting the proliferation of the secreting cells.

However, for many tissues the factors are unknown. For example, when a spleen is removed from an animal and small fragments of the spleen are transplanted into various sites in a

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splenectomized syngeneic animal, the spleen fragments grow until the integrated mass of the fragments is equivalent to that of an individual, normal spleen (Metcalf, 1964). This suggested that some factor might mediate signaling between the different spleen fragments, but the factor has not been identified. When part of the liver is removed from a mammal, the remaining liver cells begin to proliferate, and a variety of experiments indicate that a factor secreted by liver cells limits the proliferation and thus limits the size of the regenerating liver, but as above the factor remains unknown (Alison, 1986). In the phenomenon of tumor dormancy, tumors appear to secrete factors that inhibit proliferation of metastatic foci, so that when an individual has a primary tumor and metastases, surgical removal of the primary tumor appears to stimulate cell proliferation in the metastatic foci (Demicheli, 2001; Guba et al., 2001). Although the primary tumor appears to inhibit angiogenesis in the metastases (Holmgren et al., 1995), there is strong evidence that the primary tumor also secretes factors that inhibit the proliferation of single metastatic cells (Cameron et al., 2000; Guba et al., 2001; Luzzi et al., 1998). Despite the potential use of such factors to inhibit the proliferation of metastases, these factors are also unknown.

Elucidating mechanisms such as the regulation of cell proliferation can be greatly facilitated by using a simple model system such as *Dictyostelium discoideum* (Kessin, 2001). This eukaryote normally exists as vegetative amoebae that eat bacteria on soil and decaying leaves. The amoebae, which are haploid, increase in number by fission. When the amoebae are starved for bacteria, they cease dividing and begin secreting an 80 kDa glycoprotein called conditioned medium factor (CMF). When there is a high density of starving cells, as indicated by a high concentration of CMF (Jain et al., 1992; Yuen et al., 1995), the cells aggregate between 5 and 10 hours after starvation (Aubry and Firtel, 1999). The aggregating cells form large streams that break up into groups of ~20,000 cells (Shaffer, 1957). Each group develops into a fruiting body consisting of a mass of spore cells supported on a ~1 mm high column of stalk cells.

We have partially purified a secreted ~450 kDa complex of proteins called counting factor (CF) that modulates adhesion and motility during aggregation to regulate stream breakup and thus group and fruiting body size (Brock and Gomer, 1999; Gao et al., 2004; Jang and Gomer, 2005; Roisin-Bouffay et al., 2000; Tang et al., 2002). The CF preparation contains eight proteins (Brock and Gomer, 1999). To determine which of these are true components of CF and which are contaminants, we have been examining whether each protein in the preparation is part of a 450 kDa complex. In this report, we show that a 60 kDa protein is not a component of CF but part of a ~150 kDa complex, and that this protein appears to have to have the properties of a *Dictyostelium* chalone.

Materials and methods

Protein sequencing, antibody purification, western blots and immunofluorescence

Isolation of partially purified CF and SDS-polyacrylamide gel electrophoresis was done as previously described (Brock and Gomer, 1999). Tryptic digestion and sequencing of fragments of the upper band of the doublet at 60 kDa was carried out at the Baylor College of Medicine core facility. Potential glycosylation sites were identified using algorithms at http://www.cbs.dtu.dk/services/YinOYang/ and http://www.cbs.dtu.dk/services/NetNGlyc/.

A peptide corresponding to amino acids 14–34 of AprA was synthesized and used by Bethyl Laboratories (Montgomery, TX) to produce affinity-purified rabbit polyclonal anti-AprA antibodies. Staining of western blots was carried out according to Brock et al. (Brock et al., 2002) using the affinity-purified anti-AprA antibodies at 0.4 μg/ml. Staining of western blots with anti-CF50 and anti-countin antibodies was performed as previously described (Brock et al., 2002). For immunofluorescence, 200 μl of cells at 1×10^5 cells/ml were placed in the well of a 177402 eight-well slide (Nalge Nunc, Naperville, IL) and grown overnight. Cells were fixed by adding to the culture 200 μl of 2% formaldehyde in HL5. After 40 minutes the fixative was removed and cells were rinsed with TBST [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.005% Tween 20] followed by a 15 minute incubation with TBST/1% Triton X-100. Staining was carried out with 12 μg/ml affinity-purified anti-AprA antibodies in TBST and all washes with TBST following Gomer (Gomer, 1987).

Cell culture and sieving chromatography

Cell culture was carried out according to Brock et al. (Brock et al., 1999) using the wild-type Ax2 strain. Conditioned starvation medium (CM) and conditioned growth medium were prepared and concentrated according to Brock et al. (Brock et al., 2002). For growth in submerged unshaken culture, cells were grown in type 3003 tissue culture dishes (Falcon, Franklin Lakes, NJ). For size fractionation, 0.3 ml of concentrated conditioned medium was loaded on a 24 ml bed volume Superose 12 10/300 GL gel filtration chromatography column (Amersham, Piscataway, NJ), which was run at 0.3 ml/minute in PBM [20 mM KH_2PO_4, 0.01 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 6.1) with KOH], collecting fractions every minute. To assess the effect on proliferation, 5 μl of the fractions were added to 1.25×10^4 wild-type cells in 500 μl of HL5 in the well of a type 353047 24-well plate (Becton Dickinson, Franklin Lakes, NJ), and cells were counted 24 hours later. Photography of 48 hour aggregates was performed as described by Brock et al. (Brock et al., 2002). Doubling times were calculated using DT=t ln(2)/ln(fd/sd) where DT is the doubling time, ln is the natural logarithm, t is the time interval, fd is the final density and sd is the starting density. Cell viability was determined by videomicroscopy of cells (Tang et al., 2002).

cDNA isolation, sequencing, and generation of *aprA*⁻ cells

To generate a gene disruption construct, PCR was carried out on Ax2 genomic DNA with the primers CGATAATCATCCGCGGCTCCATTGGATGATTATGTC and GCATGCTCTAGAGTCCAACCTCTCTATGATGCACC, yielding a 1060 bp fragment of the 5′ side of *aprA*. This was digested with *Sac*II and *Xba*I, and ligated into the same sites in pBluescript SK+ (Stratagene, La Jolla, CA) to generate pAprA-L. PCR was then carried out with GCCAATGTAAGCTTGGACCACAAATGGTAGAATTAGC and CGCATTGGGCCCCTATATTGTAATAGTGAATCAATAGAG on Ax2 genomic DNA to generate a 1123 bp fragment of the 3′ side of *aprA*. The fragment was digested with *Hin*dIII and *Apa*I, and ligated into the same sites in pAprA-L to generate pAprA-LR. The construct pAprA-LR was digested with *Xba*I and *Hin*dIII, blunt-ended, dephosphorylated, and then the 1.4 kb *Sma*I cre-lox blasticidin resistance cassette from pLPBLP (Faix et al., 2004) was ligated into pAprA-LR to generate pAprA-KO. This was digested with *Sac*II and *Apa*I, and the insert was purified by gel electrophoresis and a Geneclean II kit (Qbiogene, Carlsbad, CA). *Dictyostelium* Ax2 cells were transformed with the construct following Shaulsky et al.

(Shaulsky et al., 1996). Five clones with the same phenotype were isolated; all of the results show data from clone DB60T3-8.

Expression of AprA in *aprA*⁻ cells

Two constructs were made to express AprA. To make an expression construct for AprA fused to a C-terminal myc tag, a PCR reaction was carried out using a vegetative cDNA library and the primers GCGCCGGTACCATGTCAAAATTATTAATTTTATTG and CGCTCGAGTTAAAGTTGCAGTTGAACTAGCAC to generate a fragment of the *aprA*-coding region corresponding to the entire polypeptide starting with the first methionine, with a *Kpn*I site on one side and an *Xho*I site on the other. After digestion with *Kpn*I and *Xho*I, the PCR product was ligated into the corresponding sites of pDXA-3D (Ehrenman et al., 2004) to produce an overexpression construct with a C-terminal *Myc* tag. *Dictyostelium aprA*⁻ cells were transformed following Manstein et al. (Manstein et al., 1995), and expression of AprA was verified by staining western blots of whole cell lysates with anti-AprA antibodies. The resulting strain was designated *aprA*⁻/*actin15*::*aprA-myc*. Approximately 100 clones with the same phenotype were isolated, and clone HDB60TOEmyc2 was used for this study. The second construct was made as above with the exception that the reverse primer was GGTCTAGATTATAAAGTTGCAGTTGAACTAGC. A TAA stop codon was incorporated at the end of the coding region and the enzyme site was changed to *Xba*I to eliminate the C-terminal *Myc* tag. This strain was designated *aprA*⁻/*actin15*::*aprA* and clone HDB60TOE9S was used. Both of the above strains synthesized and secreted AprA equally well.

Immunoprecipitation

To immunoprecipitate AprA, 1 mg of the affinity-purified anti-AprA antibodies was conjugated to 1 ml of cyanogen bromide-activated Sepharose 4B (Sigma, St Louis, MO) following the manufacturer's directions. HL5 growth medium (100 ml) conditioned by *aprA*⁻ cells or *aprA*⁻/*actin15*::*aprA* cells was concentrated as described above to 1 ml and incubated with 300 μl of the antibody resin overnight at 4°C with gentle rotation. The beads were collected by centrifugation at 5000 ***g*** for 10 seconds and washed in 20 mM sodium phosphate (pH 6.5). The beads were washed five times by resuspension in 1 ml of the sodium phosphate buffer followed by centrifugation and eluted with PBS-100 mM glycine (pH 4.0). A Profound c-Myc Tag IP/Co-IP Kit (Pierce, Rockport, IL) was used to purify AprA-myc from concentrated conditioned HL-5 following the kit protocol for functional applications. The immunoprecipitated AprA or AprA-myc was dialyzed in a Spectrapor 12–14 kDa cutoff membrane (Spectrum, Rancho Dominguez, CA) against HL5 before use in proliferation assays. Protein was quantitated by electrophoresis along with a series of BSA standards, followed by Coomassie staining and densitometry. To determine if immunoprecipitated AprA could rescue the phenotype of *aprA*⁻ cells, *aprA*⁻ cells were inoculated at 1×10^5 cells/ml in HL5 containing 10 ng/ml of immunoprecipitated AprA, and then starved on filter pads soaked with PBM containing 10 ng/ml immunoprecipitated AprA.

Cell mass, protein content, and DAPI staining

The approximate mass per 10^7 cells was calculated by measuring the mass of a pellet of 5×10^7 vegetative cells, the volume was calculated by marking the pellet, removing the cells, recounting them, filling the tube with water to that level and weighing the tube, and the protein content of a pellet of washed cells was measured using a Biorad (Hercules, CA) protein assay. The values were then divided by 5 to obtain values for 10^7 cells. The cell volumes invariably correlated with the cell masses, and indicated a density in the cell pellets of 1.02 g/ml for all cell lines. To stain nuclei, log-phase cells in HL5 were diluted to 2×10^5 cells/ml with HL5 and 200 μl was placed on a glass coverslip. After 1 hour, the medium was removed and cells were fixed with 70% ethanol at room temperature and air-dried. Vectashield/DAPI (25 μl) (Vector, Burlingame, CA) was used to simultaneously stain and mount the coverslip on a slide. For each assay, at least 200 cells were examined by epifluorescence with a 60× 1.4 NA lens.

Spore viability

To measure the viability of spores, cells were starved on filter pads as previously described (Brock et al., 1996) using 1 ml of cells at 1×10^7 cells/ml in PBM. After 5 days, the filter pad was placed in a 50 ml tube and washed repeatedly with 2 ml of PB (20 mM potassium phosphate, pH 6.2) to remove cells. All procedures were at room temperature. 0.8% v/v Nonidet P-40 alternative (2 ml) (Calbiochem, La Jolla, CA) in PB was then added to the tube (Good et al., 2003). The tube was rocked gently for 10 minutes and the filter was then removed. PB (11 ml) was then added and the cells were collected by centrifugation at 330 ***g*** for 10 minutes. The cells were then washed twice by centrifugation in 15 ml of PB. The cells were resuspended in 5 ml of PB and dissociated by trituration with a syringe and an 18 gauge needle. The density of ovoid phase-dark spores was then counted with a hemacytometer. Serial dilutions of the spores in PB were then plated with *K. aerogenes* bacteria on SM/5 plates and the number of colonies was counted 9 days later.

Results

Identification of AprA, a secreted protein that is not a CF component

We identified in the partially purified CF a 60 kDa protein we named, on the basis of the observations below, AprA for autocrine proliferation repressor (we formerly called this Ncf60 for not counting factor or 60T). We obtained amino acid sequences of three tryptic peptides of AprA and the exact matches of these were found in the predicted amino acid sequence encoded by *aprA* (GenBank Accession Number AY750687; Fig. 1) from the *Dictyostelium* genomic and cDNA sequencing projects. There are four other genes in the *Dictyostelium* genome encoding predicted proteins with 38–41% identity over the full length of the predicted AprA sequence. AprA has 28% identity and 45% similarity to PqaA, an uncharacterized hypothetical *Salmonella* protein of undetermined function, and in a region of 37 amino acids from amino acid 136 to 172 has 37% identity and 59% similarity to a 100% conserved domain in a set of three different human putative proteins of unknown function. One (AAH35817) is expressed in lymphomas; one (BAA92109) is expressed in placenta; and the third (BAC04710) is expressed in liver. The first 18 amino acids of AprA resemble a signal sequence. AprA has a putative ATP/GTP binding site motif and putative

N- and O-linked glycosylation sites. Western blots of sieving gel column fractions of conditioned starvation medium stained with affinity-purified rabbit anti-AprA antibodies as well as antibodies against CF components show that AprA elutes at ~150 kDa, while CF components elute at ~ 450 kDa (Fig. 2). These results suggest that AprA is part of a novel factor that is distinct from CF.

AprA is secreted by growing and starving cells, and affects development

To elucidate the function of AprA, we used homologous recombination to disrupt the *aprA* gene to generate *aprA*$^-$ cells. The disruption construct was engineered to replace 165 bp of the *aprA*-coding region with a blasticidin-resistance cassette. Disruption of *aprA* was verified by PCR, northern blots and western blots (Fig. 3; data not shown). Developing *aprA*$^-$ cells form large abnormal structures (Fig. 4). Videomicroscopy of developing cells indicated that whereas wild-type aggregation streams fragmented into groups, the *aprA*$^-$ aggregation streams did not fragment and coalesced into a large group. The *aprA*$^-$ phenotype was successfully rescued by expressing AprA under control of the *Dictyostelium* actin15 promoter (this causes expression during growth and early development) in the *aprA*$^-$ cells (Fig. 4, right panel). Western blots stained with affinity-purified anti-AprA antibodies indicated that the *aprA*$^-$ cells lack AprA and that the *aprA*$^-$*/actin15::aprA* cells have slightly higher levels of AprA than wild-type cells (Fig. 3A). AprA is present in growing cells and in conditioned growth medium, and in lower concentrations in conditioned starvation medium (Fig. 3A, Fig. 5). Immunofluorescence also indicated that *aprA*$^-$ cells lack AprA and that the *aprA*$^-$*/actin15::aprA* cells have more AprA than do wild-type cells (Fig. 3B). For the wild-type cells, there was no discernable cell-to cell variation in the amount or distribution of AprA in cells. Deconvolution microscopy indicated that all of the wild-type (and the *aprA*$^-$*/actin15::aprA*) cells contained AprA in a punctate distribution and in a distribution near the cell periphery (Fig. 6; data not shown). These data indicate that the phenotype of *aprA*$^-$ cells is due specifically to disruption of *aprA*, and that *aprA*$^-$ cells do not express AprA.

AprA represses the proliferation of growing cells

We noticed that the *aprA*$^-$ cells grew faster than parental cells. Proliferation curves for cells growing in liquid shaking culture starting at 2×10^5 cells/ml indicated that *aprA*$^-$ cells proliferate faster than wild-type cells and reach stationary phase at a higher cell density, while *aprA*$^-$*/actin15::aprA* cells proliferate slower and reach stationary phase at a lower density (Fig. 7A). During the first 2 days, the average doubling times were 12.7 hours for wild-type cells, 9.1 hours for *aprA*$^-$ cells and 23.3 hours for *aprA*$^-$*/actin15::aprA* cells. When this experiment was carried out with cells starting at 1×10^3 cells/ml, during the first 2 days there was no significant difference in the growth rate of the three cell lines, suggesting that at low cell densities where only very low levels of secreted factors can accumulate, there was no effect of disrupting *aprA* on proliferation (data not shown). Videomicroscopy of more than 100 cells of each of the three cell lines taken from cultures at 2×10^6 cells/ml indicated that all the cells were motile, that they extended and retracted pseudopodia, and that they had vesicles moving inside the cells, indicating that all the cells were viable. This then indicated that the differences in the growth rates of the cells at this density was not due to differences in cell viability. When grown on bacteria, *aprA*$^-$ cells also proliferated faster

than wild-type cells, and *aprA*$^{-}$*/actin15::aprA* cells were slower (Fig. 7B). Forty-eight hours after plating with bacteria, compared with wild type there were ~10-fold more *AprA*$^{-}$ cells and one tenth the number of *aprA*$^{-}$*/actin15::aprA* cells. Between the 24 and 48 hour timepoints, the doubling times were 4.3 hours for wild-type cells, 3.0 hours for *aprA*$^{-}$ cells and 9.7 hours for *aprA*$^{-}$*/actin15::aprA* cells. The observed doubling times for wild-type cells in shaking culture and on bacteria are similar to what has been observed previously (Loomis, 1982).

Wild-type but not aprA^{-} cells secrete a ~150 kDa factor that represses cell proliferation

To determine if AprA functions as a secreted signal, we used sieving gel chromatography to fractionate growth medium conditioned by growing wild-type and *aprA*$^{-}$ cells and assayed the fractions for their effect on cell proliferation. A western blot of the fractions stained with anti-AprA antibodies showed a peak of AprA at ~150 kDa in the medium conditioned by wild-type cells, and no AprA in the fractions from the *aprA*$^{-}$ cells (data not shown). There was no obvious effect of any of the *aprA*$^{-}$ fractions (assaying the material in the 450 kDa to 68 kDa range) on cell proliferation, while material at 150 kDa from the wild-type cells inhibited cell proliferation (Fig. 8). Although cells proliferate more slowly in stationary submerged culture than in shaking culture, the ~150 kDa material from wild-type cells slowed the proliferation further. The inhibition of cell proliferation by the ~150 kDa material from growth medium conditioned by wild-type cells, and no effect of fractions from medium from *aprA*$^{-}$ cells, was also seen when the fractions were added to cells in shaking culture. These data suggest the possibility that AprA is part of a 150 kDa secreted factor that inhibits proliferation.

AprA is an extracellular signal that slows proliferation

To determine if AprA functions as an extracellular signal, we isolated AprA from conditioned growth medium by immunoprecipitation and applied the immunoprecipitated material to growing cells. As shown in Fig. 9, the material collected by immunoprecipitation from *aprA*$^{-}$*/actin15::aprA* cells shows a predominant band at 60 kDa, and staining of a western blot of this material with anti-AprA antibodies indicated that it contains AprA. A similar immunoprecipitation using growth medium conditioned by *aprA*$^{-}$ cells yielded no detectable band at 60 kDa and as expected no AprA was detected on a western blot of the immunoprecipitate (Fig. 9). When added to growing wild-type or *aprA*$^{-}$ cells, the AprA immunoprecipitated from *aprA*$^{-}$*/actin15::aprA* growth medium slowed proliferation, while equal volumes of the material immunoprecipitated from *aprA*$^{-}$ growth medium had no discernable effect on proliferation (Table 1). We observed that 1 ng/ml AprA had no significant effect on cell proliferation, whereas 2 and 5 ng/ml AprA partially inhibited proliferation compared with 10 ng/ml AprA. AprA concentrations up to 100 ng/ml gave approximately the same amount of proliferation inhibition as 10 ng/ml, suggesting that there is a maximum amount by which AprA can slow proliferation. Similar results were obtained using a Myc-tagged AprA isolated by immunoprecipitation with an anti-Myc antibody (data not shown). Together, the data suggest that the increased proliferation rate of *aprA*$^{-}$ cells is due to the lack of extracellular AprA, and that AprA functions as an extracellular signal to repress proliferation.

AprA also helps to coordinate cytokinesis with mitosis

While examining *aprA*$^{-}$ cells by optical microscopy, we noticed significantly more cells in the *aprA*$^{-}$ population with two, three or more nuclei. This was also seen when staining the nuclear DNA with DAPI (Fig. 10). Counts of DAPI-stained cells showed that, compared with wild-type cells, the *aprA*$^{-}$ population contained significantly more cells with two nuclei, while the *aprA*$^{-}$/*actin15*::*aprA* population contained significantly fewer cells with two nuclei (Table 2). In addition, the *aprA*$^{-}$ population also contained significantly more cells with three or more nuclei compared with wild-type and *aprA*$^{-}$/*actin15*::*aprA* cells (Table 2). We occasionally observed cells with as many as four nuclei in wild-type and three nuclei in *aprA*$^{-}$/*actin15*::*aprA* cells, whereas *aprA*$^{-}$ cells were observed with as many as eight nuclei. When grown on a surface in unshaken submerged culture, there was also an abnormally high number of multinucleate *aprA*$^{-}$ cells compared with similarly cultured wild-type cells. As proper cytokinesis should limit the number of nuclei in a cell to a maximum of two, the data suggest that AprA is required for proper cytokinesis.

On a per nucleus basis, AprA does not affect growth

The growth (the increase in mass or protein per hour) and the proliferation (the increase in the number of cells per hour) of cells can be regulated independently (Dolznig et al., 2004; Gomer, 2001; Jorgensen and Tyers, 2004; Saucedo and Edgar, 2002). To determine whether AprA regulates growth as well as proliferation, we measured the mass and protein content of populations of cells. For cells growing in shaking culture, the average mass of *aprA*$^{-}$ cells was slightly higher than the average mass of wild-type cells, and the average amount of protein in *aprA*$^{-}$ cells was slightly less than that of wild-type cells (Table 2). The values for wild-type cells are in agreement with previously reported values (Ashworth and Watts, 1970). The *aprA*$^{-}$/*actin15*::*aprA* cells had, on average, more mass and protein. However, *aprA*$^{-}$ cells had more nuclei per 100 cells compared with wild type, whereas *aprA*$^{-}$/*actin15*::*aprA* cells had fewer (Table 2). After normalizing to the number of nuclei, on average *aprA*$^{-}$ cells have less mass and protein per nucleus than wild-type cells, and *aprA*$^{-}$/*actin15*::*aprA* cells have more mass and protein per nucleus than either *aprA*$^{-}$ or wild-type cells (Table 2). As cells will roughly double their mass in one doubling time, a rough estimate of the growth rate can be obtained by dividing the cell mass or protein content by the doubling time. On a per cell basis, *aprA*$^{-}$ cells accumulate more mass and protein per hour than wild-type cells, while *aprA*$^{-}$/*actin15*::*aprA* cells accumulate less mass and protein per hour (Table 3). When the growth was calculated per nucleus, there was no significant difference in the mass accumulation rate between *aprA*$^{-}$ and wild type, while the mass accumulation rate was lower for *aprA*$^{-}$/*actin15*::*aprA* (Table 3). For all three cell lines, the protein accumulation rate per nucleus was approximately the same (Table 3). Together, the data suggest that although cells that lack *aprA* proliferate faster and on a cell basis accumulate more mass and protein than do wild-type cells, the increased growth rate is due to the increased proliferation and is not due to an increased mass or protein accumulation per nucleus.

The drawback to not having AprA is a decreased ability to form spores

A very puzzling aspect of the *aprA* gene is that disrupting it results in cells that proliferate faster. One would thus think that not having this gene would give an evolutionary advantage to cells. As the phenotype of the aggregates formed by *aprA*$^-$ cells is different from that of wild-type cells, we examined the possibility that cells having an intact *aprA* gene might have some advantage over *aprA*$^-$ cells after the cells have starved. One of the most important aspects of *Dictyostelium* development is that some of the cells form spores. Thus, to assess one measure of fitness, we measured the percentage of cells that could form spores. We found that *aprA*$^-$ cells as well as *aprA*$^-$/*actin15*::*aprA* cells had a reduced number of cells that formed visible as well as viable spores (Table 4). These data indicate that abnormally low or high levels of AprA reduce the ability of cells to form spores, and that cells thus need an optimal amount of AprA for efficient spore formation.

Discussion

Although there is a considerable amount of data supporting the idea that chalones exist, in most cases the signals have not been identified (Gamer et al., 2003; Gomer, 2001). We have found that AprA is secreted by *Dictyostelium* cells and represses their proliferation without affecting their viability. This indicates that AprA is a chalone. However, we were unable to completely inhibit proliferation by adding immunoprecipitated AprA, so it is possible that, unlike theoretical chalones, AprA can only slow but cannot stop proliferation. As the factor containing AprA is ~150 kDa and AprA is 60 kDa, it is unclear if this factor is a multimer of AprA or consists of AprA and other proteins.

At first glance, it seems odd that *Dictyostelium* cells would deliberately slow their proliferation (for cells forming a specific tissue in a higher eukaryote, however, there is a clear advantage to the whole organism for a specific tissue to not grow beyond its appropriate size). Because we observe AprA being produced by and then affecting cells that were cloned from a single cell and have not yet begun to differentiate, and that all cells contain AprA in a similar distribution and thus potentially all cells secrete AprA, we consider AprA as having an autocrine effect. The fact that a secreted factor is being used suggests that as the cell density and number increase, the concomitant increase in the concentration of the proliferation-repressing factor would slow proliferation more at high cell density than at low density. This indicates that for *Dictyostelium*, there is an evolutionary advantage to slowing proliferation when cells begin to get crowded. We have found that when equal numbers of wild-type and *aprA*$^-$ cells are starved, the *aprA*$^-$ cells produce fewer spores than wild-type cells, suggesting that AprA potentiates normal development. It thus appears that AprA represses proliferation because this confers an advantage to the population of cells. There is an obvious evolutionary advantage to efficient spore formation, so `cheater' mutants that do not respond to AprA and thus proliferate faster would presumably have reduced spore formation and thus would be at a disadvantage, as is the case with *dimA*$^-$ cells, which do not respond to the DIF signal (Foster et al., 2004)

Comparing *aprA*$^-$, wild-type and *aprA*$^-$/*actin15*::*aprA* cells, we observe that increasing amounts of AprA correlate with a decrease in the number of cells with two nuclei. Assuming that cells cannot significantly shorten the time it takes to undergo cytokinesis, this might

suggest that as AprA levels increase there are fewer cells undergoing cytokinesis, which would then qualitatively correlate with the division times of the three cell lines. Disruption of *aprA* also leads to an increase in the percentage of cells with three or more nuclei. Mutations in several genes necessary for cytokinesis in *Dictyostelium* also result in multinucleate cells, although these cells can have up to 50 nuclei per cell (Adachi, 2001). It is interesting that several different human tumor types have multinucleate cells (Jayaram and Abdul Rahman, 1997; Long and Aisenberg, 1975; Nonomura et al., 1995; Ramos et al., 2002). AprA thus effectively has two functions: the first to slow the cell cycle and the second to coordinate cytokinesis with mitosis.

In addition to inhibiting cell proliferation, AprA also appears to reduce the net mass and protein accumulation of a population of cells (Table 2). Thus, by one criterion AprA inhibits growth as well as proliferation. However, when we normalized the mass and protein accumulation on a per nucleus basis, we observed that the *aprA*$^{-}$ population accumulates mass at the same rate as wild-type cells. Abnormally high levels of AprA, however, did inhibit mass accumulation on a per nucleus basis. One possible explanation for this is that there is an upper limit to the size of a cell (Grewal and Edgar, 2003; Mitchison, 2003; Saucedo and Edgar, 2002), so that when the slowly proliferating *aprA*$^{-}$/*actin15*::*aprA* cells reach a certain size they stop accumulating mass. Also on a per nucleus basis, abnormally high or low levels of AprA do not significantly affect the rate of protein accumulation. With the exception of the effect of abnormally high levels of AprA on mass accumulation, AprA does not affect growth on a per nucleus basis. Assuming that each nucleus can drive mass and protein accumulation at a fixed rate, this indicates that the effect of AprA on growth can be attributed solely to its effect on cell proliferation.

For the *aprA*$^{-}$ cells, the connection between faster proliferation during growth phase and the formation of larger structures when the cells starve and consequently develop is unclear. However, alterations in the metabolism of growing *Dictyostelium* cells affects structure size during development; for example, increasing intracellular glucose levels causes cells to form larger fruiting bodies (Garrod and Ashworth, 1972). As *aprA*$^{-}$ cells proliferate faster than wild-type cells, a reasonable conclusion is that the *aprA*$^{-}$ cells have a different composition than wild-type cells; for example, we observe that *aprA*$^{-}$ cells have less mass and protein per nucleus than wild-type cells. Our working hypothesis is that the altered group size, abnormal structures and reduced spore viability observed in *aprA*$^{-}$ cells are in part a secondary consequence of the effect of AprA on repressing the proliferation of growing cells.

We do not know the signal transduction pathway that cells use to sense AprA. Three *Dictyostelium* transformants, *crlA*$^{-}$, *yakA*$^{-}$ and *qkgA*$^{-}$, have phenotypes resembling that of *aprA*$^{-}$ in that they proliferate faster and reach a higher stationary phase density than parental cells. In addition, *crlA*$^{-}$ and *qkgA*$^{-}$ cells, like *aprA*$^{-}$ cells, also form abnormally large structures. CrlA has similarity to seven-transmembrane G-protein-coupled cAMP receptors (Raisley et al., 2004). YakA is a kinase, and appears to stop growth in response to stresses such as starvation, and thus regulates the growth to development transition (Taminato et al., 2002). The predicted QkgA amino acid sequence contains a predicted kinase domain (Abe et al., 2003). It is thus possible that some of the associated proteins may be part of the AprA

signal transduction pathway, and that similar proteins may be components of chalone signal transduction pathways in higher eukaryotes.

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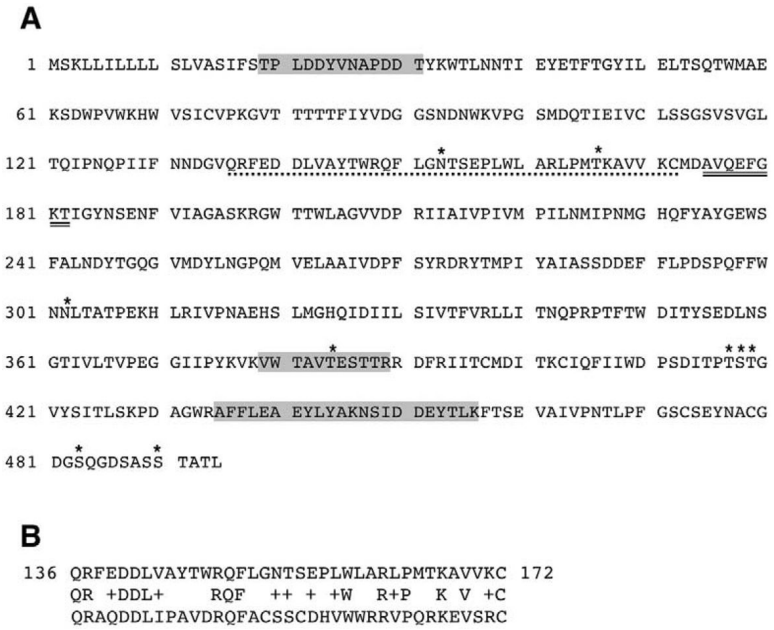

Fig. 1.

(A) The predicted amino acid sequence of AprA. The amino acid sequences from tryptic peptides of AprA are shown by grey boxes. The SignalP program predicted that amino acids 1–18 are a signal sequence, with a maximum cleavage site probability (0.933) between amino acids 18 and 19. Asterisks indicate potential N-and O-linked glycosylation sites. A double underline indicates a potential ATP-GTP binding site. The broken underline indicates the region with some similarity to human proteins. (B) The partial sequence of AprA (top line) compared with a short region of similarity in three different human ESTs.

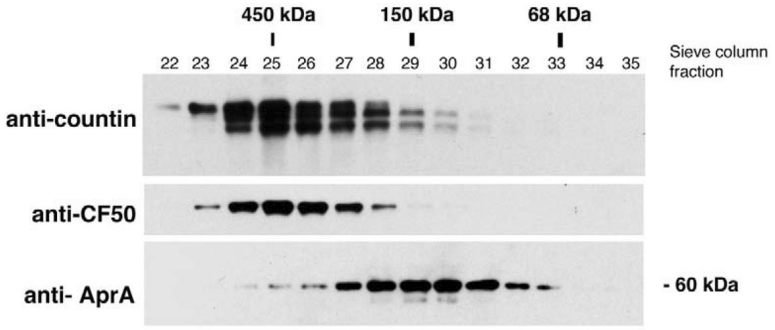

Fig. 2.

AprA is secreted by starving *Dictyostelium* cells, but is not part of the CF complex. Conditioned starvation medium from wild-type cells was fractionated by sieving gel filtration chromatography. Western blots of the fractions were stained with affinity-purified rabbit antibodies against CF50, countin and AprA. On the SDS-polyacrylamide gels, the upper band stained with anti-countin antibodies is at 40 kDa, the CF50 band is at 50 kDa and the heavy AprA band is at 60 kDa. The affinity-purified anti-AprA antibodies stained a band at ~56 kDa in fractions 28–30. Besides this band and the heavy band at 60 kDa, the anti-AprA antibodies did not stain any other band. See Fig. 3 for validation that the anti-AprA antibodies are staining the product of the *aprA* gene.

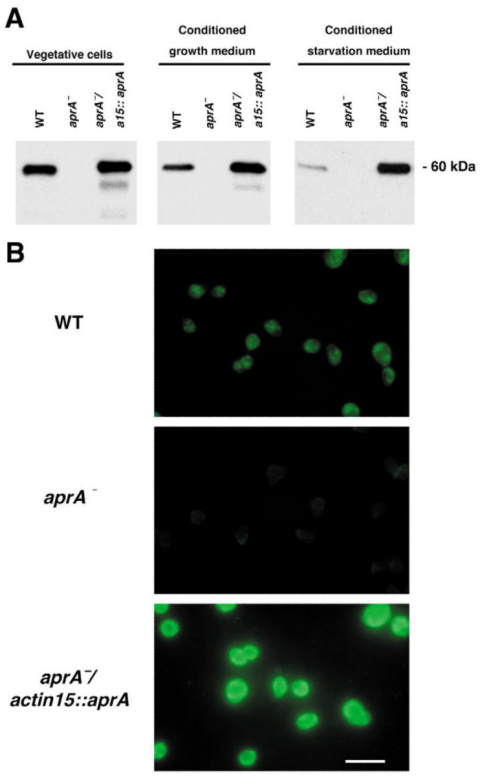

Fig. 3.

AprA is present in and is secreted by vegetative cells. (A) Cells growing in shaking culture (vegetative cells), conditioned growth medium, and conditioned starvation buffer were electrophoresed on an SDS-polyacrylamide gel. A western blot of the gel was stained with affinity-purified anti-AprA antibodies. `*aprA*⁻/*a15*::*aprA*' indicates *aprA*⁻/*actin15*::*aprA*. (B) Growing cells were stained for AprA by immunofluorescence. Scale bar: 20 μm.

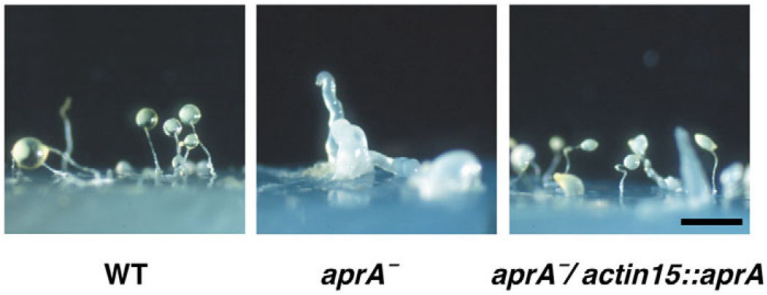

Fig. 4.

Disruption of *aprA* by homologous recombination causes cells to form large abnormal structures during development. The phenotype can be rescued by expression of the *aprA* cDNA under control of an actin15 promoter (right panel). WT indicates the wild-type parental cells. Scale bar: 0.5 mm.

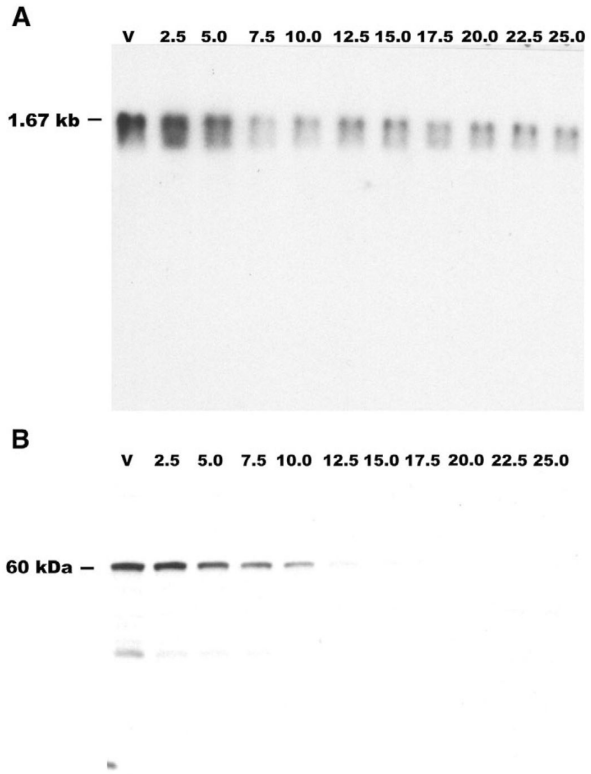

Fig. 5.

AprA is expressed in vegetative cells and during early development. Ax2 wild-type cells were starved on filters and samples were harvested at the indicated hours after starvation according to Brock et al. (Brock et al., 2002). (A) A Northern blot of RNA extracted from cells harvested at the indicated times in hours after starvation (V indicates vegetative cells) was probed for *aprA* mRNA. (B) A western blot of whole cell lysates from cells harvested at the indicated times was stained with anti-AprA antibodies.

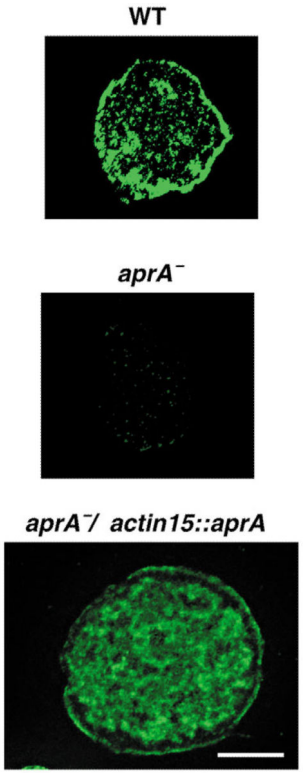

Fig. 6.

The subcellular distribution of AprA. Cells from preparations similar to those shown in Fig. 3B were imaged with a Zeiss Axioplan II deconvolution microscope. Scale bar: 5 μm.

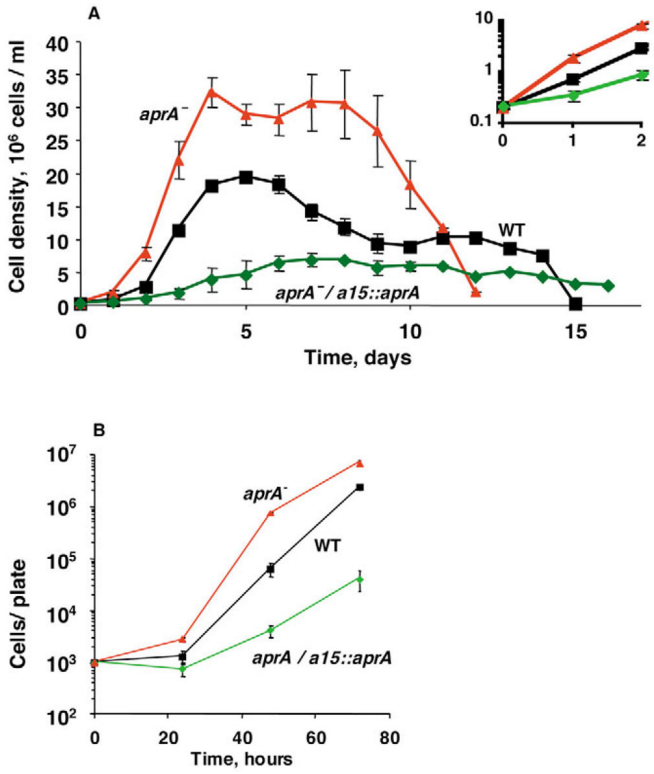

Fig. 7.

AprA slows the proliferation of cells. (A) Cells were diluted to 2×10^5 cells/ml in HL5 and the cell density was measured daily. The graph shows means±s.e.m. from three independent experiments. WT indicates wild type. The insert shows the data from the first two days plotted using a log scale for the density. The saturation densities (in units of 10^7 cells/ml) were 3.24±0.06 for *aprA*⁻, 1.94±0.07 for wild type and 0.69±0.10 for *aprA*⁻/*actin15*::*aprA*. The differences between the wild-type saturation density and either the *aprA*⁻ or the *aprA*⁻/*actin15*::*aprA* saturation density were significant ($P<0.01$; one-way ANOVA; Dunnett's test). (B) For each cell type, three 100 mm petri dishes with SM/5 agar were spread with a lawn of bacteria mixed with 1000 *Dictyostelium* cells. At the indicated times, the bacteria and cells were washed off one of the plates, and the *Dictyostelium* cells were counted. The graph shows means±s.e.m. from three independent experiments. At 72 hours, the difference between wild type and *aprA*⁻/*actin15*::*aprA* was significant ($P<0.05$) and the difference between wild type and *aprA*⁻ was significant ($P<0.01$; one-way ANOVA, Dunnett's test). The absence of error bars indicates the error was smaller than the plot symbol.

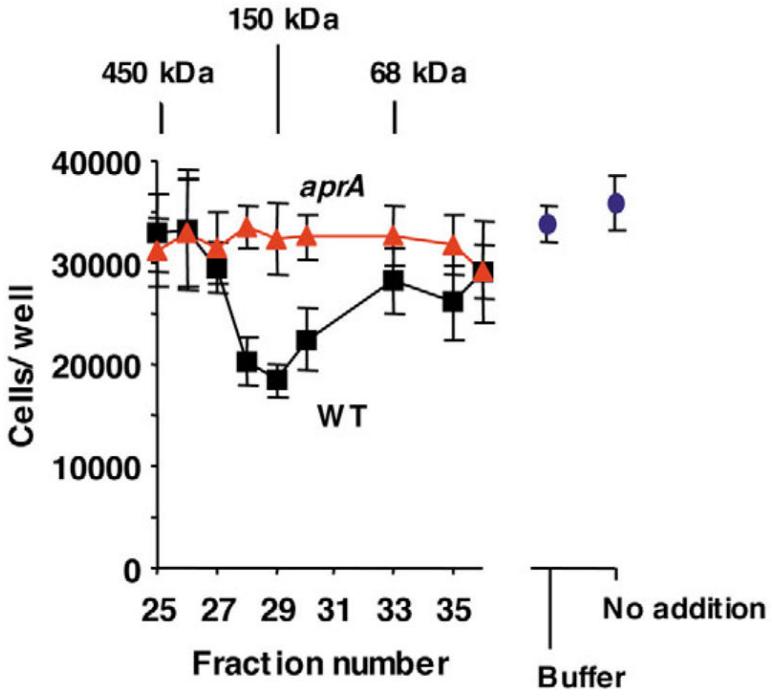

Fig. 8.

Growing wild-type cells secrete a ~150 kDa factor that represses cell proliferation, and *aprA*$^{-}$ cells do not accumulate this factor. Conditioned growth media from the indicated cell types were fractionated on the Superose gel filtration column used for Fig. 1. Indicated fractions (5 μl), as well as 5 μl of the column buffer (Buffer) were added to wild-type cells, and the cells were counted 24 hours later. Values are means±s.e.m. from three independent assays. The difference between the counts for fraction 29 of the exudates from wild type and *aprA*$^{-}$ is significant ($P<0.025$; *t*-test).

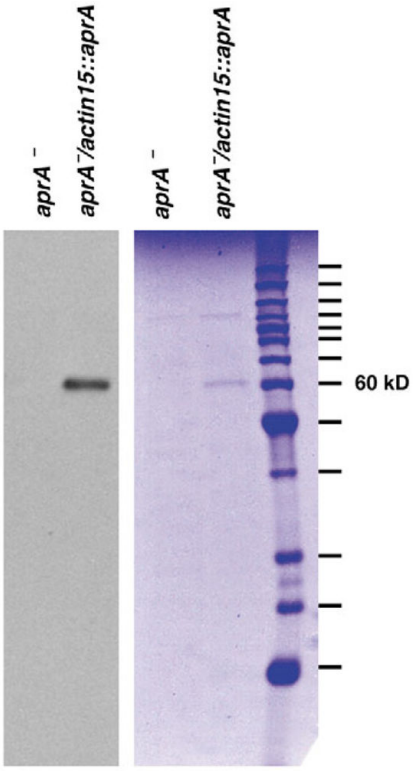

Fig. 9.

Purification of AprA by immunoprecipitation. AprA was immunoprecipitated from growth medium conditioned by the indicated cell types and electrophoresed on an SDS-polyacrylamide gel and stained with Coomassie blue (left panel). A western blot of a similar gel was stained with anti-AprA antibodies (right panel).

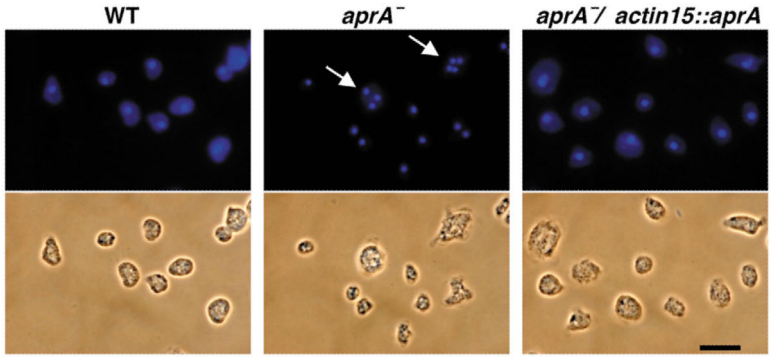

Fig. 10.

AprA helps to prevent the formation of multinucleate cells. (Top row) Cells growing in shaking culture were fixed with ethanol and stained with DAPI to show nuclear DNA (the background staining in the cells is mitochondrial DNA). The bottom row shows phase contrast images of the same cells. Multinucleate cells (white arrows) are present in the *aprA*⁻ population. Scale bar: 20 μm.

Table 1

Exogenous AprA inhibits cell proliferation

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

Cells were inoculated at 1×10^5 cells/ml in shaking culture, and immunoprecipitated AprA or an equal volume of the material immunoprecipitated from *aprA*$^-$ cells (control) was added. The cell density was then determined 24 hours later. Values are means±s.e.m. from three separate experiments. The effect of AprA on wild-type cells is significant (P<0.03) and the effect of AprA on *aprA*$^-$ cells is significant (P<0.01; t-tests).

Table 2

The effect of AprA on the mass and protein content of cells

	Per 10^7 cells		Percent of cells with n nuclei				Per 10^7 nuclei	
Cell type	**Mass (mg)**	**Protein (mg)**	**1**	**2**	**3 +**	**Nuclei/100 cells**	**Mass (mg)**	**Protein (mg)**
Wild type	12.3±0.3	0.44±0.01	74±1	24±1	2±1	129±1	9.5±0.2	0.34±0.01
aprA $^-$	13.2±0.1	0.41±0.01	41±2	44±4	15±3	190±8	6.9±0.3	0.21±0.02
aprA$^-$/*actin15*::*aprA*	14.0±0.2	0.66±0.01	92±2	7±2	1±1	108±2	12.9±0.3	0.61±0.01

The mass and protein content of cells was measured as described in the Materials and methods, and the percent of cells with 1, 2, 3 or more nuclei was measured by counts of DAPI-stained cells. After calculating the average number of nuclei per 10^7 cells, the mass and protein per 10^7 nuclei was calculated. All values are means±s.e.m. from three independent assays. The difference in the percentage of cells with two nuclei between any two cell lines was significant ($P<0.05$), the difference in the percentage of cells with three or more nuclei between *aprA*$^-$ and either of the other two cell lines was significant ($P<0.01$), and the difference in the percentage of cells with three or more nuclei between wild-type and *aprA*$^-$/*actin15*::*aprA* cells was not significant (one-way ANOVA, Tukey's test).

Table 3

The effect of AprA on the mass and protein increase of cells

	Per 10^7 cells per hour		Per 10^7 nuclei per hour	
Cell type	**Mass (mg)**	**Protein (μg)**	**Mass (mg)**	**Protein (μg)**
Wild-type	0.96±0.02	35±1	0.75±0.02	27±1
aprA $^-$	1.45±0.01	45±1	0.76±0.03	24±1
aprA$^-$/*actin15*::*aprA*	0.60+0.01	28±1	0.55±0.01	26±1

The mass and protein values shown in Table 1 were divided by the observed doubling times to obtain the approximate increases in mass and protein content per hour. For the increase in mass per 10^7 nuclei per hour, the difference between *aprA*$^-$/*actin15*::*aprA* and either of the other two strains is significant (P<0.01), while the difference between wild type and *aprA*$^-$ is not significant (one-way ANOVA, Tukey's test). Forthe increase in protein per 10^7 nuclei per hour, the difference between any two strains is not significant (one-way ANOVA, Tukey's test).

Table 4

The effect of AprA on the ability of cells to form spores

Cell type	Percent of cells forming visible spores	Percent of cells forming detergent-resistant spores
Wild type	80±3	28±8
aprA $^{-}$	42±16	5±2
aprA^{-}/*actin15*::*aprA*	36±12	2±1

Cells were starved on filter pads, and after fruiting bodies had formed, cells were dissociated and the number of phase-dark spores was counted. The cells were incubated with detergent to kill any vegetative or prestalk cells (a large fraction of spores are resistant to detergent; stalk cells are already dead), and plated with bacteria. The number of colonies was counted 7 days later. The percentage of the original 10^7 cells forming visible as well as detergent-resistant viable spores was then calculated. Values are mean±s.e.m. from three separate experiments.