

eIF2 α Kinases Control Chalone Production in *Dictyostelium discoideum*[∇]

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Growing *Dictyostelium* cells secrete CfaD and AprA, two proteins that have been characterized as chalones. They exist within a high-molecular-weight complex that reversibly inhibits cell proliferation, but not growth, via cell surface receptors and a signaling pathway that includes G proteins. How the production of these two proteins is regulated is unknown. *Dictyostelium* cells possess three GCN2-type eukaryotic initiation factor 2 α subunit (eIF2 α) kinases, proteins that phosphorylate the translational initiation factor eIF2 α and possess a tRNA binding domain involved in their regulation. The *Dictyostelium* kinases have been shown to function during development in regulating several processes. We show here that expression of an unregulated, activated kinase domain greatly inhibits cell proliferation. The inhibitory effect on proliferation is not due to a general inhibition of translation. Instead, it is due to enhanced production of a secreted factor(s). Indeed, extracellular CfaD and AprA proteins, but not their mRNAs, are overproduced in cells expressing the activated kinase domain. The inhibition of proliferation is not seen when the activated kinase domain is expressed in cells lacking CfaD or AprA or in cells that contain a nonphosphorylatable eIF2 α . We conclude that production of the chalones CfaD and AprA is translationally regulated by eIF2 α phosphorylation. Both proteins are upregulated at the culmination of development, and this enhanced production is lacking in a strain that possesses a nonphosphorylatable eIF2 α .

The translational initiation factor eukaryotic initiation factor 2 (eIF2) functions to deliver initiator tRNAs to ribosomes that are assembling on mRNAs. The delivery, and thus the efficiency of translation, is regulated by phosphorylation of the α subunit of eIF2 (eIF2 α) at serine 51 (11). While phosphorylation of eIF2 α may decrease translation in a general manner, often it enhances the translation of specific mRNAs (5, 10, 16, 17, 19, 31) by a mechanism using short upstream open reading frames (18). There are four different types of eIF2 α -specific kinases found in mammals, GCN2, PERK, HRI, and PKR (11). PERK is also found in all multicellular eukaryotes, while the GCN2 kinase is found in yeast and fungi, *Dictyostelium*, and many other eukaryotes (11). These kinases are activated by various cellular and environmental stimuli through the four types of regulatory domains that control the activity of the kinase domain. Translational regulation mediated by the four types of eIF2 α kinases is involved in many cellular responses and processes, including the unfolded-protein response and endoplasmic reticulum stress (PERK); cell cycle progression and viral defense (PKR); iron/heme limitation (HRI); and a variety of stresses, including general nutrient limitation and amino acid starvation (GCN2) (11, 18). Among other recent studies of the functions associated with translational regulation by eIF2 α phosphorylation are those demonstrating roles in controlling cell proliferation (4, 5, 20, 30, 39).

Dictyostelium cells have three initiation factor kinases, IfkA, IfkB, and IfkC, that are homologs of GCN2 and thus sense amino acid deprivation. Three other, uncharacterized genes

have kinase domains similar to those of eIF2 α kinases but lack regulatory domains similar to one of the four types described above. Studies on the expression of the *ifkA*, *-B*, and *-C* genes show that all three are expressed during growth and throughout development (14, 29). *In situ* hybridizations demonstrate a high degree of spatially restricted expression for each gene during development (29). Studies using disruptions of the *ifk* genes have indicated that IfkA functions in modulating the timing of aggregation and early gene expression, that IfkB functions in maintaining proper prestalk-specific gene expression, and that IfkA and IfkB function in maintaining proper cell-cell and cell-substrate adhesion and the equilibrium between different cell types for proper spatial patterning (14, 29).

Recent studies have characterized two proteins of *Dictyostelium*, CfaD and AprA, that act as chalones, i.e., endogenous proliferation inhibitors involved in reversibly regulating tissue size (7, 37). CfaD and AprA are secreted during growth and are part of an extracellular 150-kDa complex that represses proliferation but does not affect cell growth (1, 6). A receptor for AprA has been characterized (8), and the signaling pathway downstream of the chalone receptor involves G proteins (2) and a ROCO family kinase (26). While there is some understanding of the mechanism of action of CfaD and AprA in inhibiting proliferation, nothing is known about the control of their production. We demonstrate here that eIF2 α phosphorylation regulates the production of the two chalone proteins.

MATERIALS AND METHODS

Generation of BS167. To replace the endogenous eIF2 α gene with a copy carrying a mutation in codon 51 that results in an S51A mutated protein product, a knock-in construct was made as follows. (In *Dictyostelium* eIF2 α , the residue equivalent to the canonical S51 phosphorylation site of yeast GCN2 [10] is S53. Here, we refer to this residue generically as S51, given the prevalent and general use of the yeast numbering to identify the residue phosphorylated by eIF2 α kinases.) A 1,875-bp fragment of the promoter and the entire coding region was amplified from genomic DNA using primers if2-5 (5') and if2-2 (3') (Table 1)

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
if2-4.....	5' ATAGATCTGATACGACGACGTGCAATTTTC 3'
if2-5.....	5' AGGGCCACTGGTTAATGCTGTAAAAATTG 3'
if2-2.....	5' GGATCCTTATTTTCTGCTCGATTTCTTTTCC 3'
if2-1.....	5' GGATCCTAAAAATGGTATTCGAAAAATGATTG 3'
ifkA-9.....	5' GGATCCGGAGGTTCAATGTTTATAGATATCATTCTAGA 3'
ifkA-10.....	5' CTCGAGTTAAAAACAATTTCTCCATCAA 3'
ifkA-13.....	5' GGAGTATTGTTCAAAGGGGAACCTTGAAAAAC 3'
ifkA-14.....	5' AAAACCAGCCAATATTTTAATCTTGATAATG 3'
AprA-5.....	5' GAACCACTTTGGTTAGCAAG 3'
AprA-3.....	5' CCCATTAAAGAGTGTTTCAG 3'
CfaD-5.....	5' GTAGTCACCCAGATGTTTAC 3'
CfaD-3.....	5' ACCAAAAGTCCAACATGAAC 3'

and cloned into the pGEM T-easy vector (Promega). Mutagenesis of the S51 codon was carried out as described previously (32) employing the if2-4 oligonucleotide (Table 1), which alters the serine codon to one encoding alanine. A blasticidin resistance gene cassette (bsr) (35) was attached at the end of the coding region. A 1,350-bp fragment of the coding region amplified from genomic DNA with if2-1 and if2-2 primers (Table 1) was cloned to the opposite side of the bsr cassette, resulting in the knock-in construct pif2 α -25. The second coding region also possessed the S51A mutation made as described above. pif2 α -25 was digested to release the eIF2 α -bsr DNA, and this was transformed into Ax4 cells using electroporation (33). Genomic DNA was isolated from blasticidin-resistant clones possessing insertions at the endogenous eIF2 α gene (determined by PCR screening) and used as a template in a PCR to amplify a 1,000-bp fragment spanning the proximal promoter and coding region past the S51 codon. PCR products were sequenced to identify clones in which double-crossover products occurred, so as to replace the S51 codon with the A51 codon. In such clones, there is an intact eIF2 α promoter and a coding region possessing the S51A mutation. Independent isolates gave the same phenotypes, and one such strain was named BS167 and was used for the experiments described in this paper.

Generation of GyrB/kinase domain fusions. To generate an inducible, active kinase domain, the *ifkA* kinase domain was fused to the GyrB domain of the *Escherichia coli* gyrase. This GyrB domain contains an ATPase active site that can be inhibited by coumermycin, causing dimerization of the domain and any fused polypeptides (15, 36). A 1,896-bp fragment of the *ifkA* coding region was amplified from genomic DNA using oligonucleotides ifkA-9 and ifkA-10 (Table 1), with flanking BamHI and XhoI sites derived from the ends of the primers. This represents amino acids 881 to 1514, which align with the kinase domain of yeast GCN2. The fragment was cloned into the pGEM T-easy vector. Activating mutations based on studies with yeast GCN2 mutations (28) were made as described above employing primers ifkA-13 and ifkA-14 (Table 1). The double-mutated kinase domain possessed a K1258G and an F1320L change. An EcoRI/BamHI 690-bp fragment from pC939 (36) and coding for residues 1 to 220 of the *E. coli* GyrB protein (its dimerization domain) was fused to the actin 15 promoter in the plasmid Exp4(+) (13). The BamHI/XhoI kinase domain fragments were subsequently fused to the 3' end of the GyrB coding region, resulting in pifkA-6 (wild-type kinase domain) and pifkA-14 (double-mutant kinase domain). Each plasmid was transformed into various strains using electroporation. In each instance, at least two independent populations of transformed cells were used in independent experiments.

Cell growth and conditioned medium. Cells were grown in HL-5 medium (9) at 21°C. The titers of the cells were determined by direct counting with a hemocytometer. When present, coumermycin was added to a final concentration of 1 μ g/ml from a stock of 5-mg/ml coumermycin in DMSO (dimethyl sulfoxide). For collection of conditioned medium, cultures were started at 5×10^5 cells per ml and grown for 48 h or to the desired cell titers. Coumermycin was added for 12, 24, or 48 h, and the cells were removed by centrifugation for 4 min at $5,000 \times g$. The supernatant was removed and spun for 5 min at $9,000 \times g$ to ensure removal of all cells. The supernatant from the second spin was either used immediately or frozen at -80°C .

Protein synthesis measurements. Cell cultures at a titer of 3×10^6 cells per ml were split into two subcultures, one with and one without coumermycin at 1 μ g/ml. The titers of cells were determined at 4 and 24 h after addition to monitor the effect, if any, of coumermycin on proliferation. To minimize any manipulation-induced stress on the cells that might affect translation, 5 ml of cells after 4 h of growth with or without coumermycin treatment was removed and placed in a new flask. After being shaken for 5 min, 25 μ l of 10 mCi/ml Tran³⁵S-label (MP

Biomedicals) was added to each shaking flask. At 0, 5, 10, 15, and 20 min; a 1-ml sample was removed; and the cells were rapidly pelleted (15 s), washed with ice-cold PDF (22 mM potassium phosphate, pH 6.5, 20 mM KCl, 5 mM Mg₂Cl) containing unlabeled methionine, and taken up in 75 μ l of protein lysis buffer (200 mM NaPO₄, 20 mM KCl, 5 mM MgCl₂, 5% [wt/vol] sucrose, 0.5% NP-40 at pH 7.5). Samples were incubated at room temperature for 5 min and clarified by centrifugation for 5 min at $8,000 \times g$. The supernatant was collected and stored at -80°C .

To separate labeled proteins from amino acids, 15 μ l of the above-mentioned samples was spotted on squares of Whatman chromatography paper and submerged in ice-cold 10% trichloroacetic acid (TCA) for 15 min. The filters were transferred to boiling 5% TCA for 10 min and then once again transferred to ice-cold 5% TCA for 10 min. The filters were washed once with ethanol, followed by an ether wash. The filters were dried, and the radioactivity was determined by scintillation counting.

Protein isolation and Western blotting. Conditioned medium obtained as described above was concentrated 10-fold using Amicon Ultracel-10K filtration units, and 20- μ l samples were electrophoresed on NuPage Bis-Tris gels (Invitrogen). Western analysis was carried out using either anti-Countin antibodies at a dilution of 1:500, anti-AprA at 1:1,000, or anti-CfaD at 1:3,000. The antibodies were generously provided by Richard Gomer.

For isolation of extracellular protein during development, the method described by Bakthavatsalam and Gomer (3) was used. After removal of bacteria by differential centrifugation, 2×10^7 cells, harvested from growth on bacteria, were placed on type 353102 1- μ m-pore-size polyethylene terephthalate membrane six-well format cell culture inserts (Becton Dickinson). The liquid was removed after the cells had settled, and 0.45 ml of PDF was added to the wells so that the liquid just touched the membrane. At various times poststarvation, the conditioned buffer was removed from a well and stored at -80°C . After collection at all time points, the samples were lyophilized and reconstituted to 0.06 ml, and 15 μ l of each sample was electrophoresed and subjected to Western analysis.

RT-PCR. RNA was isolated using Trizol (Invitrogen). Reverse transcription (RT)-PCR was carried out as described previously (25) using either *cfaD*- or *aprA*-specific oligonucleotides (Table 1). Oligonucleotides specific for the H7 gene were used as an internal control (40).

Spore viability analysis. Spore-filled sori from Ax4 and BS167 cells growing on bacteria were picked and resuspended in PDF. After two washes in PDF, the titer of the spores was determined, and they were resuspended in 10 mM EDTA, pH 7.5, 0.1% NP-40. The samples were incubated at 42°C for 45 min, followed by two washes with PDF. The spores were resuspended with *Klebsiella pneumoniae* and plated at 100 spores per plate onto SM plates (34). Colonies, derived from individual spores, were counted after incubation for several days at 21°C.

RESULTS

Ectopic activation of Ifks inhibits proliferation. eIF2 α kinases require dimerization for activation (27, 36). In order to examine the functions of the initiation factor kinases in *Dictpostelium*, the kinase domains of the three kinases were fused to the chemically inducible dimerization domain of the GyrB protein of *E. coli*. The drug coumermycin induces dimerization of the GyrB protein and, thus, polypeptides fused to the

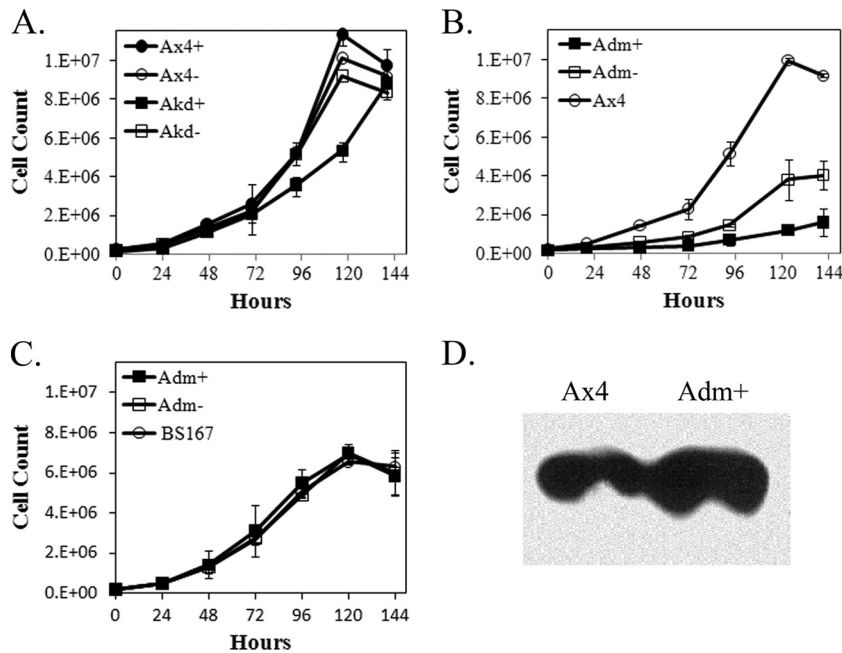


FIG. 1. Effects on proliferation by ectopic expression of active kinase domains. Cultures were started at 1.5×10^5 cells per ml, and the cells were counted every 24 h. At least three independent experiments were performed for each strain. The error bars indicate SEM. (A) Ax4 cells and Ax4 cells transformed with *pifkA-6* (Akd, wild-type *IfkA* kinase domain fused to *GyrB*). +, coumermycin, -, no coumermycin. For clarity, the error bars for only the Ax4⁺ and Akd⁺ cells are shown; the differences seen at 93 and 117 h are significant ($P < 0.05$; Student's *t* test). (B) Ax4 cells and Ax4 cells transformed with *pifkA-14* (Adm, double-mutant kinase domain fused to *GyrB*). The differences between Ax4 and Adm⁻ and Ax4 and Adm⁺ are significant at 20 h and all subsequent time points ($P < 0.01$). The differences between Adm⁻ and Adm⁺ are significant at 47 h and all subsequent time points ($P < 0.05$). (C) BS167 cells (possessing S51A eIF2 α) and BS167 cells transformed with *pifkA-14* (Adm). There were no significant differences at any time points. (D) Western analysis of extracellular Countin produced in Ax4 cells or Ax4 cells transformed with *pifkA-14* (Adm). Cultures started at 5×10^5 cells per ml were grown to 3×10^6 cells per ml, followed by the addition of coumermycin for 12, 24, or 48 h. Cells were removed from the medium, and the medium was used in the Western analysis. Shown are the results from 24 h plus coumermycin conditioned medium.

dimerization domain (15, 36). Fusions were made using the normal kinase domain coding regions, as well as coding regions that had been mutated in residues demonstrated in yeast GCN2 to be activating mutations of the kinase activity (28). Initial characterizations indicated that ectopic activation of the kinase domains during growth inhibited proliferation. The extent of inhibition was dependent upon which of the three kinases was used, the presence or absence of activating mutations, and the presence or absence of coumermycin, presumably reflecting differences in the intrinsic activities of the kinase domains separated from their regulatory domains. The *IfkA* fusions were chosen for more detailed examination.

Ax4 cells transformed with *pifkA-6* (*GyrB* fused to the *IfkA* kinase domain, referred to here as Akd) resulted in no effect on proliferation relative to untransformed Ax4 cells unless coumermycin was added to the culture (Fig. 1A). Coumermycin addition resulted in a slight but reproducible decrease in the proliferation rate. In contrast, cells transformed with *pifkA-14* (*GyrB* fused to the *IfkA* kinase domain possessing two activating mutations, referred to here as Adm, for double mutant of *IfkA*) resulted in severe inhibition of proliferation, even without coumermycin (Fig. 1B). Addition of coumermycin resulted in a further reduction in proliferation, and most often addition resulted in only 2 to 4 cell doublings, followed by little to no further proliferation. The enhanced effect on proliferation by the Adm fusion protein relative to the unmu-

tated Akd fusion indicates that the activating mutations that were patterned on the yeast GCN2 protein do activate the *Dictyostelium* *Ifks*.

To demonstrate that the effect on proliferation was due to eIF2 α phosphorylation by the kinase domain fusions, the plasmids were transformed into BS167. This strain is a knock-in strain that possesses an eIF2 α gene in which the serine codon corresponding to the phosphorylation site has been mutated to an alanine codon. No effect on cell proliferation was seen in BS167 cells transformed with the kinase fusion plasmids, with or without coumermycin addition (Fig. 1C). This indirect method of demonstrating that dependence of the inhibitory effect on proliferation was due to phosphorylation of eIF2 α was necessitated by the fact that no phospho-eIF2 α -specific antibodies currently exist for the *Dictyostelium* protein. Further confirmation that the kinase domain fusions phosphorylate eIF2 α was obtained by demonstrating that the protein Countin was induced by the presence of the fusion proteins (Fig. 1D). It was previously shown that eIF2 α phosphorylation by *IfkA* enhances the production of Countin (14).

Ectopic activation of *Ifks* does not result in general inhibition of translation. The severe inhibitory effect on proliferation by the expression of the activated kinase domain of *IfkA* might be due to a general shutdown or severe reduction in protein synthesis as a result of substantial phosphorylation of eIF2 α . Such an effect is seen in yeast when an active PKR is

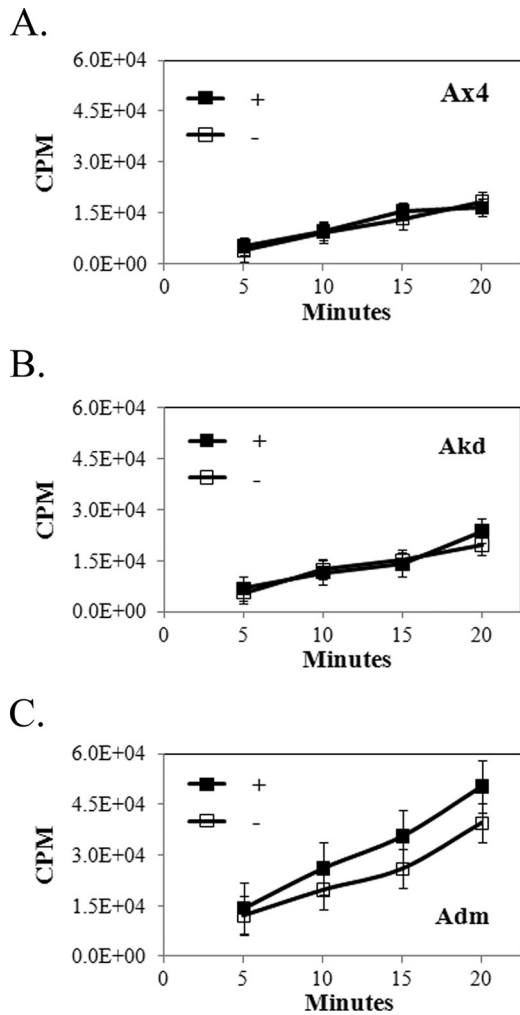


FIG. 2. Effect of ectopic expression of active kinase domains on overall protein synthesis. The incorporation of radioactive methionine (CPM) in Ax4 cells (A), Ax4 cells transformed with pifkA-6 (B), and Ax4 cells transformed with pifkA-14 (C) in the presence (+) or absence (-) of coumermycin is shown. Three independent trials were performed. The error bars indicate SEM. No significant differences were found with or without coumermycin in any of the strains (Student's *t* test).

overexpressed (36). The relative rates of protein synthesis in the presence and absence of coumermycin were examined in Ax4 cells and Ax4 cells transformed with the Akd and Adm constructs. Cells were counted for a 24-h period to confirm the effects on proliferation shown in Fig. 1. The cells were taken from the culture medium (with or without coumermycin) and incubated with radioactive methionine. Incorporation levels were measured over a 20-min period. No significant differences were found in the presence versus absence of coumermycin, importantly, even in cells expressing the Adm fusion kinase domain (Fig. 2). Overall incorporation varied somewhat from strain to strain, and the presence of the double-mutant kinase domain repeatedly gave higher incorporation, a finding that remains unexplained. Silver staining of gels of total protein isolated from Ax4 cells expressing or not expressing the Adm fusion protein in the presence of coumermycin indicated no significant or reproducible differences in the total protein patterns.

Ectopic activation of Ifks inhibits proliferation through a secreted factor. To determine if the inhibitory effect on proliferation was cell autonomous or due to a secreted factor, conditioned medium was obtained from Ax4 cells expressing the Adm fusion kinase domain and growing in medium containing coumermycin for 12, 24, or 48 h. Untransformed Ax4 cells were grown in normal medium to a titer of ca. 3×10^6 cells per ml. The culture was split into two groups of cells, and both were pelleted. One was resuspended at the initial titer in Adm conditioned medium, while the other was resuspended at the initial titer in the same medium in which the cells were previously growing. As seen in Fig. 3A, conditioned medium inhibited cell proliferation. The maximal extent of inhibition was found using conditioned medium obtained after 24 h of growth in the presence of coumermycin. The effect was reversible, as Adm cells growing with coumermycin that were resuspended in fresh medium showed a burst of proliferation prior to slowing to the initial low rate (Fig. 3B).

Ectopic activation of Ifks induces CfaD and AprA production. Previous studies characterized two chalcones in *Dictyostelium*, AprA and CfaD, that are secreted during growth and that reversibly inhibit proliferation (1, 6, 8). The production of

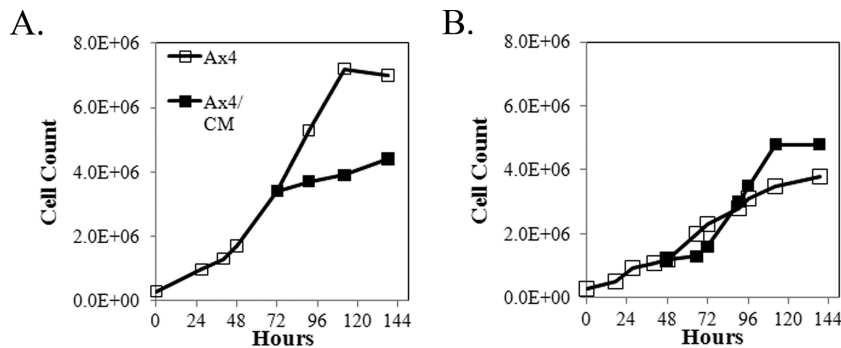


FIG. 3. Inhibition of proliferation by ectopic expression of the active kinase domain is due to a secreted factor(s). (A) Conditioned medium was obtained by starting cultures of Ax4 cells transformed with pifkA-14 at 5×10^5 cells per ml and growing them to 3×10^6 cells per ml. Coumermycin was added for 24 h, and the culture medium was cleared of cells. Conditioned medium was added to half of the cells from a growing Ax4 culture to give the same titer as the other half of the culture, and the cell count was examined for another 72 h. The result shown is one of two trials, both of which resulted in inhibition of proliferation. (B) Reversibility of the inhibition of proliferation by coumermycin. Adm cells were grown in the absence of coumermycin (open squares). At 48 h, coumermycin was added to half of the culture (solid squares), resulting in inhibition of proliferation. After a further 24 h, the cells were transferred to fresh medium without coumermycin, resulting in enhanced proliferation.

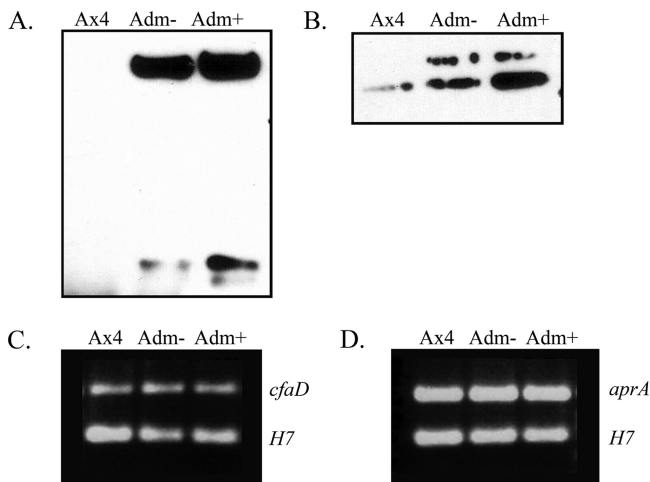


FIG. 4. Western analysis of extracellular CfaD and AprA, and RT-PCR on their mRNAs during ectopic expression of the active kinase domain. Conditioned medium was collected from Ax4 cells and Ax4 cells transformed with *pifkA-14* in the presence (+) or absence (-) of coumermycin. (A) The 58.5- and 27-kDa forms of CfaD detected using anti-CfaD antiserum. (B) The 60-kDa band of AprA detected using anti-AprA antiserum. The faint upper band was sometimes seen and may represent a modified version of AprA. RNA was isolated from the indicated cells in the presence (+) or absence (-) of coumermycin. (C) RT-PCR was carried out with primers specific for *cfaD* (upper band) and for the *H7* gene (lower band) as an internal control. (D) RT-PCR was carried out with primers specific for *aprA* (upper band) and for the *H7* gene (lower band) as an internal control.

these two proteins was examined in Ax4 cells transformed with the Adm plasmid. The conditioned medium after growth with or without coumermycin was collected and used in Western analysis. Extracellular AprA and CfaD were found at much higher levels when the cells were expressing the Adm fusion protein (Fig. 4A and B). The levels were even higher when coumermycin was present in the culture medium.

To examine *aprA* and *cfaD* mRNA levels, RT-PCR was carried out on RNA isolated from Ax4 cells and Ax4 cells transformed with the Adm plasmid growing in the presence or absence of coumermycin. In contrast to the greatly enhanced production of the chalone proteins, the corresponding mRNA

levels were unchanged in cells expressing the Adm fusion protein (Fig. 4C and D), and they remained unchanged when coumermycin was included in the medium.

These results suggest that the inhibition of proliferation caused by the presence of the activated IfkA kinase domain is due at least in part to enhanced production of extracellular AprA and CfaD. To confirm involvement in the inhibition by the two chalone, the Adm plasmid was transformed into the null strains of *aprA* and *cfaD*. Proliferation of the transformants was compared to that of the respective untransformed null strains in the presence and absence of coumermycin. Expression of the Adm protein had no effect on proliferation for *cfaD*-null cells with or without the presence of coumermycin (Fig. 5A). A somewhat lower rate of proliferation was seen in *aprA*-null cells when they expressed the Adm fusion protein, but the cells did reach a titer at saturation similar to that of the untransformed cells (Fig. 5A). The inhibitory effect is much less pronounced than that seen in Ax4 cells (compare Fig. 1B). Importantly, the inhibition was not enhanced by the presence of coumermycin. That little to no difference in proliferation was found in the chalone-null strains suggests the inhibitory effect is mostly attributable to the overproduction of extracellular AprA and CfaD when the IfkA kinase domain is ectopically active in growing cells that contain the corresponding genes.

Lack of increase in chalone production during development in BS167 cells. Enhanced production of extracellular AprA and CfaD by ectopic phosphorylation of eIF2 α inhibits cell proliferation. While these results demonstrate a competency for regulating chalone production by eIF2 α phosphorylation, they do not reveal when such regulation might normally come into play during growth and/or development of *Dictyostelium* cells. One possible scenario might be to regulate cell proliferation during development. Development occurs during amino acid starvation, which should be sensed by the Ifks, and previous work demonstrated that IfkA phosphorylates eIF2 α during development (14). Whether and how much cell division occurs during development is somewhat controversial. Recent experiments indicate that a low and decreasing percentage of cells divide during the first 9 h of development, followed by a burst of division during the next 3 h while mounds form tips (24).

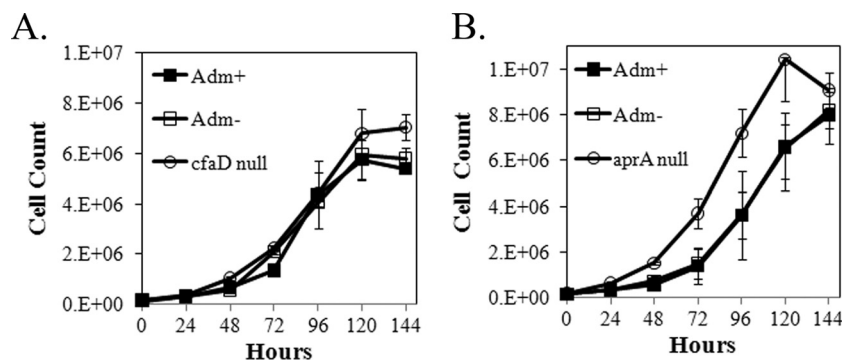


FIG. 5. Ectopic expression of the active kinase domain in strains that lack either CfaD or AprA. The *cfaD*- and *aprA*-null strains were transformed with *pifkA-14*. Cell proliferation was followed in the presence (+) and absence (-) of coumermycin. Three independent experiments were performed. The error bars indicate SEM. The difference seen at the 144-h time point between *cfaD*-null cells and *cfaD*-null cells expressing Adm was significant ($P < 0.05$; Student's *t* test). In panel B, differences between transformed (both with and without coumermycin) and untransformed *aprA*-null cells at the 48-h through the 120-h time points were significant ($P < 0.05$).

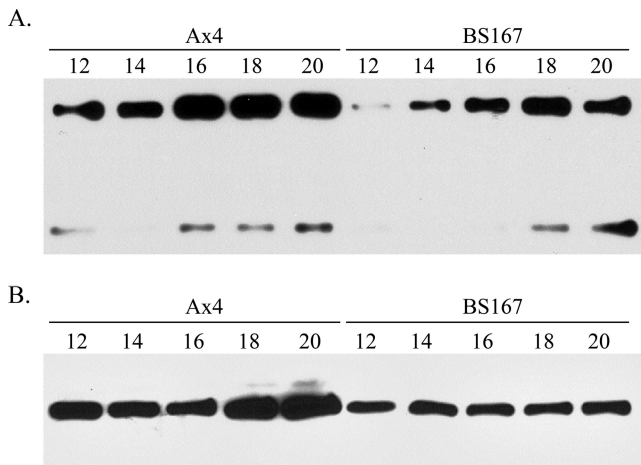


FIG. 6. Western analysis of extracellular CfaD and AprA during development in the Ax4 and BS167 strains. Cells growing in the presence of bacteria were harvested and plated for development in a manner that allows easy recovery of secreted proteins. Samples were collected at the indicated times (in hours poststarvation). After concentration, the samples were subjected to Western analysis. (A) The 58.5- and 27-kDa forms of CfaD detected using anti-CfaD antiserum. (B) The 60-kDa band of AprA detected using anti-AprA antiserum.

The number of cells undergoing division subsequently drops rapidly to very low numbers during the remaining stages of finger formation and culmination (24). We wondered if chalone production functions at these times to influence proliferation during development.

Ax4 and BS167 cells were plated for development in a manner that facilitated the isolation of secreted proteins (3). Several time points were examined by Western analysis for CfaD and AprA production. Little to no protein could be reliably detected during the first half of development. Both proteins were detected once mounds had formed (10 to 12 h poststarvation) and at subsequent times. In developing Ax4 cells, CfaD levels increased from 12 to 20 h poststarvation (Fig. 6A), during which time tipped mounds transitioned to fingers that then underwent culmination. This corresponds to the time at which Muramoto and Chubb found decreasing numbers of dividing cells (24). In developing BS167 cells, which possess an eIF2 α that cannot be phosphorylated (S51A mutation), there was much less CfaD protein present at 12 h, and only a modest increase occurred, so that the level at 20 h was roughly equivalent to that at 12 h in Ax4 cells (Fig. 6A). Similar results were found for AprA. Increased production with time of development was seen in Ax4 cells, with little to no increase found in the BS167 strain, and the levels at 20 h in BS167 cells were less than those at 12 h in Ax4 cells (Fig. 6B).

BS167 spores are not viable. One characteristic of the *aprA*- and *cfaD*-null strains is the production of spores with reduced viability—about 44% that of wild type spores for *cfaD*-null cells (1) and 18% of wild-type viability for *aprA*-null cells (6). It was thus of interest to examine spore viability in the BS167 strain. After growth on bacteria and sporulation, Ax4 and BS167 spores were collected and assayed for viability; 43% \pm 2% of Ax4 spores were viable, while only 3% \pm 2% of BS167 spores were viable (\pm standard error of the mean [SEM]; $n = 4$).

Morphologically, the BS167 spores were atypical in that they were round instead of oval, as is found for wild-type spores.

DISCUSSION

Expression of the unregulated and active kinase domain of IfkA severely inhibited proliferation of *Dictyostelium* cells. Inhibition of proliferation was due to phosphorylation of eIF2 α , as demonstrated by a lack of inhibition in cells possessing an eIF2 α that could not be phosphorylated (S51A mutation) and by the increased production of Countin, a protein previously demonstrated to be upregulated by a shift of its mRNA onto ribosomes upon eIF2 α phosphorylation (14). No decrease in overall protein synthesis was detected. Thus, the inhibitory effect was not due to a gross repression of translation, suggesting instead a specific effect on activation of one or more mRNAs that produce proteins that inhibit proliferation.

Conditioned medium obtained from the kinase domain-expressing cells was found to inhibit the proliferation of nonexpressing cells. Greatly enhanced production of extracellular AprA and CfaD was found in the conditioned medium. Extracellular CfaD is found in several sizes, with the 60- and 27-kDa polypeptides typically being the prevalent forms (1). Enhanced production of both the 60- and 27-kDa forms was found in cells expressing the activated IfkA kinase domain. While there was enhanced protein production for both chalones, the mRNA levels for *aprA* and *cfaD* essentially remained unchanged. This is consistent with an enhanced rate of translation of the two mRNAs upon phosphorylation of eIF2 α . Both genes have several small open reading frames upstream of the respective coding regions; such open reading frames are hallmarks of mRNAs upregulated by eIF2 α phosphorylation (11). Direct examination of the polysome profiles of *aprA* and *cfaD* mRNAs will need to be carried out to confirm enhanced translation brought about by eIF2 α phosphorylation.

AprA and CfaD are chalones that are found in a 150-kDa complex that inhibits proliferation, but not growth, of *Dictyostelium* cells (1, 6, 8). The presence of both is required for the inhibition of proliferation, even though it appears there may be separate receptors for the two proteins (8). Exogenously added CfaD does not decrease the proliferation rate of *aprA*-null cells (1), and likewise, added AprA does not decrease the proliferation rate of *cfaD*-null cells (8). This predicts that if the inhibitory effect of expressing the activated IfkA kinase domain is mostly due to the enhanced production of CfaD and AprA, then the effect should not be seen in either of the null strains. Indeed, expressing the activated kinase domain in *aprA*-null cells had no effect on proliferation. Expression in the *cfaD*-null cells did give a reduction in proliferation, but one that was not nearly as dramatic as that seen in Ax4 cells and that was not abrogated further by the addition of coumermycin. While most of the reduction may thus be attributable to the enhanced production of CfaD in the Adm-expressing cells, the slight but significant reduction seen may indicate the involvement of an additional, unknown factor(s).

The inhibition of proliferation by the ectopically expressed IfkA kinase domain is an artificial situation, as the phosphorylation of eIF2 α is not under normal regulation. Nonetheless, it points to a capability of controlling chalone production by eIF2 α phosphorylation. Previous work demonstrated that

eIF2 α phosphorylation occurs during the developmental program of *Dictyostelium* (14, 29). Recent studies on cell division during development demonstrated a low level of cell division occurring during early development, followed by a sizable increase in the number of cells undergoing division during tipped-mound formation (9 to 12 h poststarvation) (24). The number of cells undergoing division drops substantially at the culmination of development, at which time prestalk and prespore tissues are being formed.

Examination of the production of AprA and CfaD from 12 to 20 h poststarvation revealed an increase in the extracellular levels of both chalones in wild-type cells. This increase coincides with the time when the number of cells undergoing division drops from the highest numbers to the lowest numbers found during the developmental program (24). The increase in AprA and CfaD levels was not seen in developing cells that possessed the S51A eIF2 α that could not be phosphorylated. In this strain, the levels of both proteins at 20 h were comparable to or less than those found at 12 h in wild-type cells. These results suggest that AprA and CfaD play a role in inhibiting cell division during culmination that is mediated by eIF2 α phosphorylation, enhancing the production of the chalones during the later stages of development. The lack of inhibition of division during culmination thus predicted in the BS167 and the *aprA*- and *cfaD*-null strains might contribute to spores from these strains being nonviable. Testing this postulated function of the two chalones will require examining cell division during development in the null strains for these two genes and in the BS167 strain. Regulation of cell numbers during the stages of development when prestalk and prespore tissues are being formed would certainly substantiate the naming of AprA and CfaD as chalones.

Amino acid deprivation plays a major role in the *Dictyostelium* developmental program (21–23). That the Ifks in *Dictyostelium* are involved in chalone production and hence in regulating cell proliferation is perhaps not surprising, as these GCN2-type kinases possess a regulatory domain that senses and responds to amino acid starvation (12, 38). In mammals, kinases that phosphorylate eIF2 α have been found to affect cell proliferation by several mechanisms (4, 5, 20, 30, 39). The finding that in *Dictyostelium* chalone production is controlled at least in part by eIF2 α phosphorylation opens up the possibility that eIF2 α kinases may function similarly in regulating chalones, such as myostatin, in mammals.

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