The Putative bZIP Transcription Factor BzpN Slows Proliferation and Functions in the Regulation of Cell Density by Autocrine Signals in Dictyostelium

Jonathan E. Phillips¹, Eryong Huang², Gad Shaulsky², Richard H. Gomer³*

¹ Department of Biochemistry and Cell Biology, Rice University, Houston, Texas, United States of America, ² Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, United States of America, ³ Department of Biology, Texas A&M University, College Station, Texas, United States of America

Abstract

The secreted proteins AprA and CfaD function as autocrine signals that inhibit cell proliferation in Dictyostelium discoideum, thereby regulating cell numbers by a negative feedback mechanism. We report here that the putative basic leucine zipper transcription factor BzpN plays a role in the inhibition of proliferation by AprA and CfaD. Cells lacking BzpN proliferate more rapidly than wild-type cells but do not reach a higher stationary density. Recombinant AprA inhibits wild-type cell proliferation but does not inhibit the proliferation of cells lacking BzpN. Recombinant CfaD also inhibits wild-type cell proliferation, but promotes the proliferation of cells lacking BzpN. Overexpression of BzpN results in a reduced cell density at stationary phase, and this phenotype requires AprA, CfaD, and the kinase QkgA. Conditioned media from high-density cells stops the proliferation of wild-type but not bzpN cells and induces a nuclear localization of a BzpN-GFP fusion protein, though this localization does not require AprA or CfaD. Together, the data suggest that BzpN is necessary for some but not all of the effects of AprA and CfaD, and that BzpN may function downstream of AprA and CfaD in a signal transduction pathway that inhibits proliferation.

Introduction

During organismal development, tissues of characteristic shapes and sizes are generated from progenitor cell populations through proliferation and differentiation. Although great insights have been gained regarding the mechanisms that pattern tissues during development, much remains to be understood about how the sizes of tissues are established and maintained [1,2]. Proliferation-inhibiting signals that are produced by and act on the same population of cells can regulate the size of tissues by effectively halting proliferation once a certain cell number has been reached [3]. For instance, the TGF-β superfamily member myostatin is secreted by skeletal muscle and inhibits the proliferation of muscle cell progenitors, functioning to limit muscle size [4]. Although there is evidence of such signals operating in many cell types [5,6,7], few signaling molecules of this sort have been identified and little is known about intracellular signaling pathways that couple such signals to the inhibition of proliferation. Some tumors appear to secrete factors that inhibit the proliferation of tumor cells [8], and such factors secreted by primary tumors may inhibit the proliferation of metastases [9,10]. Thus, identification and characterization of autocrine signals that inhibit proliferation may be useful in developing therapies to limit tumor growth and to keep metastases in a dormant state.

The social amoeba Dictyostelium discoideum is an excellent model system for the study of extracellular signals that inform individual cells about local cell numbers [11,12,13]. When nutrients are abundant, Dictyostelium exists as unicellular amoebae. However, under conditions of starvation, and if the density of starving cells is above a certain threshold as indicated to the cells by high levels of CMF, a protein secreted by starving cells [12], cells secrete and respond to the chemottractant cAMP [14], leading to the formation of streams of cells that aggregate to form groups of about 20,000 cells [15]. These aggregates develop into multicellular fruiting body structures composed of differentiated cell types and consisting of an approximately 2 millimeter high stalk supporting a ball of spores [16]. The stalk structure aids in dispersal of spores to areas where nutrients are present [17], resulting in spore germination and resumption of vegetative growth.

During vegetative growth, Dictyostelium cells secrete the proteins AprA and CfaD, which inhibit the proliferation of Dictyostelium cells in a concentration-dependent manner [18,19]. 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 [18,19]. The addition of either recombinant AprA (rAprA) or rCfaD to wild-type cells slows proliferation, though cells lacking AprA are not slowed by rCfaD [19], and cells lacking CfaD are not slowed by rAprA [20], suggesting that these proteins require each other for activity. Cells lacking AprA or CfaD accumulate mass on a per nucleus basis at a rate like wild-type cells, indicating that AprA and CfaD regulate proliferation.
but not cell growth [18,19]. 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 after reaching stationary density, and that aprA and cfaD cells produce fewer viable spores than wild type following starvation-induced development [18,19].

Little is known about the signal transduction pathway downstream of AprA and CfaD. The G protein complex subunits Ga6, Ga9, and GB are necessary for the activity of AprA [21], suggesting that AprA may signal through a G protein-coupled receptor. Additionally, the ROCO kinase QkgA is necessary for the inhibition of proliferation by AprA and CfaD, and qkgA cells phenocopy the rapid proliferation, high stationary density, and multinuclearity of aprA and cfaD cells [22]. However, qkgA cells do not show defects in spore viability [22], indicating that AprA and CfaD affect spore viability by a mechanism independent of QkgA.

Basic leucine zipper (bZIP) transcription factors are a large family of proteins that function in a wide range of signal transduction pathways [23,24] and are defined by an approximately 30 amino-acid sequence with a leucine residue at every seventh position, which mediates homo- or heterodimerization through alpha-helical interactions [25] and a stretch of adjacent basic residues that mediate DNA binding [26]. The Dictyostelium genome encodes 19 predicted bZIP transcription factors. Little is known regarding the function of the majority of these bZIP proteins, though the bZIP proteins DimA and DimB are downstream effectors of the prestalk cell fate-specifying signal DIF and translocate to the nucleus in response to this signal [27].

apatkan the putative bZIP transcription factor BzpN plays a role in AprA- and CfaD-mediated inhibition of proliferation. bzpN cells proliferate rapidly, show aberrant responses to rAprA and rCfaD, and are multinucleate. However, bzpN cells do not show defects in spore viability and do not proliferate to a higher stationary density than wild type. Thus BzpN mediates a subset of the effects of AprA and CfaD.

**Materials and Methods**

Deletion mutation of bzpN and BzpN-GFP construct

Regions upstream and downstream of the bzpN gene, which flank the entire open reading frame of the gene, were PCR-amplified (primers for upstream amplification: 5′-TCCCGCGGT-TTGTGATTACGGCACAACA and 5′-TCAAGCTTCCA-GGTGATGAAGGATTGA; primers for downstream amplification: 5′-TGGTAGCAACACCGCAGCCTCTACCTTCAA and 5′-TCCCGCGCACAACCGGATTATTTCCCATTCAAACAA). Both PCR fragments were purified and digested with the restriction endonuclease SacII, and then the two fragments were ligated together and PCR was done using the primers 5′-TCCGTTACAACCGCAGCCTCTACCTTCAA and 5′-TCAAGCTTCCAAGGATTGA to amplify the ligated product. This PCR product was digested and cloned into the KpnI-HindIII sites of the pLPBLP-ΔSacII plasmid [30] to generate the null knockout vector. For transformation of AX4 cells, 10 μg of the knockout vector was linearized using SacII and then electroporated into AX4 cells [31]. Cells were selected in HL5 medium supplemented with 10 μg/ml Blasticidin S for at least 10 days. Colonies were plated along with bacteria (Klebsiella aerogenes) on SM agar plates for plaque formation. Colony PCR was performed on the resulting plaques to identify knockout mutants. Colonies that yielded a PCR product using the diagnostic primer (5′-GGCGAAAAGTTTGAA-CAACAACAA) and a BSR cassette internal primer (5′-TCTTGTGAGAATGTAAATTACATC) were chosen. The successful gene disruption was further verified by RT-PCR with the primers ATGGTAGCGGTGATACGATTG and GCAGTTTGAGCATCCAAAT, which amplified a genomic DNA fragment, and then reverse-transcribed into cDNA with an oligo-dT primer (Invitrogen). Real-time PCR was performed using a Bio rad (MJ research) Opticon3 system as described [27].

To generate a bzpN-GFP transgene, the bzpN open reading frame lacking the stop codon was amplified by PCR from wild-type vegetative cDNA with the primers 5′-GGACTAATTTGG- TATCAAGATTTGGCTACACAGG and 5′-GGAATTAAGTA- GTATAAAGTTGGATACATAGAATGAAG, which incorporate terminal SpeI sites. This PCR product was cloned into the pGM-E7 vector (Promega). The bzpN open reading frame was then isolated by SpeI digestion and gel purification, and ligated into the SpeI site of the Dictyostelium extrachromosomal expression vector pDM323 [32] to generate an open reading frame encoding BzpN with GFP fused to the C-terminus. Correct orientation of the bzpN open reading frame within the vector was confirmed by restriction mapping. This vector was transformed into Dictyostelium cells following Manstein et al [33].

**Cell culture, proliferation curves, inhibition assays, immunoblotting, mass and protein measurement, colony size measurement, and spore viability**

Cell culture, proliferation curves in shaking culture, proliferation on lawns of bacteria, and nuclei counts were done according to [18]. Comparison of extracellular AprA and CfaD by immunoblotting, measurement of mass and protein content, colony size measurement on bacterial lawns, and spore viability assays were done following [22]. Silver staining of conditioned media was done following [13]. rAprA and rCfaD inhibition assays were done following [22], except that cells were incubated with rAprA or rCfaD for 48 hours before measurement of cell density.

To measure proliferation in response to stationary phase conditioned media, wild-type cells were grown to a density of 20–25×10⁶ cells/mL in HL5. Cells were removed by centrifugation at 800×g for 3 minutes at room temperature and the conditioned medium supernatant was collected and sterilized by passage through a 0.2 micron filter (Pall, Ann Arbor, MI). Wild-type, bzpN, or bzpN/bzpN-GFP cells were then resuspended in this conditioned media in 5 μL volumes at 0.5×10⁶ cells/mL, and cell density was determined by hemocytometer after a 24 hour incubation in shaking culture at 22°C.

**Fluorescence microscopy**

To determine the effect of conditioned media on BzpN-GFP localization, conditioned media from wild-type, aprA, and cfaD cells at a density of 20–25×10⁶ cells/mL was collected as described above. bzpN/acl5:3:bzpN-GFP cells at a density of 0.5–4×10⁶ cells/mL were resuspended in 0.5 μL volumes at 0.5×10⁶ cells/mL in the media in which they had been growing, in fresh HL5, or in conditioned media from wild-type, aprC, or cfaD cells. These cultures were incubated overnight at 22°C in shaking culture, and then 200 μL of each culture was allowed to settle in one well of an 8-well chamber slide (Nunc) for 30 minutes. The medium was removed and cells were then fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 minutes, permeabilized with 0.1% NP-40 in PBS pH 7.4 for 2 minutes, and washed three times in PBS pH 7.4. Cells were then mounted in Vectashield mounting media with DAPI (Vector, Burlingame, CA) and imaged.
with an Olympus FV1000 fluorescence microscope. To examine BzpN-GFP localization in comparison to lysosomal or mitochondrial localization, bzpN−/bzpN-GFP cells at 0.5–4 x 10⁶ cells/mL in shaking culture were resuspended in low-fluorescence media [34] at 0.5 x 10⁶ cells/mL with either 500 nM Lysotracker Red dye (Invitrogen) or 500 nM Mitotracker Red dye (Invitrogen) and incubated in shaking culture at 22°C for 2 hours. Cells were then washed once in low-fluorescence media and then fixed and imaged as described above.

Results

BzpN regulates proliferation

To gain insight into the function of basic leucine zipper transcription factors encoded in the *Dictyostelium* genome, genes encoding bZIP proteins were targeted for disruption by homologous recombination [27]. The BzpN gene encodes a 999-amino acid protein that has 33% identity and 56% similarity over a 59-amino acid segment to the human bZIP protein Nrf2, which mediates cellular responses to oxidative stress [35]. A BzpN null mutant was generated and confirmed by diagnostic PCR and RT-PCR (Figure 1A). *bzpN*² cells exhibited rapid proliferation as compared to wild-type cells. To determine if the rapid proliferation was due to loss of BzpN, we constructed an extrachromosomal vector in which the constitutive actin15 promoter drives expression of BzpN with GFP fused to the C-terminus, transformed wild-type and *bzpN*² cells with this vector, and examined the proliferation of these cell lines. In shaking culture, *bzpN*⁻ cells showed faster proliferation than wild-type cells during the logarithmic phase of proliferation and had a significantly faster doubling time (Figure 1B and Table 1). However, *bzpN*⁻ cells did not proliferate to a higher cell density than wild type (Table 1), in contrast with *aprA*⁻ and *cfaD*⁻ cells [18,19]. The expression of BzpN-GFP in *bzpN*⁻ cells resulted in a proliferative phenotype and doubling time similar to wild-type cells, strongly suggesting that the rapid proliferation phenotype of *bzpN*⁻ cells is due specifically to the absence of BzpN. Although *bzpN*⁻/act15::bzpN-GFP cells did not show a reduced maximum cell density as compared to wild type, expression of BzpN-GFP in wild-type cells resulted in a significantly reduced maximum cell density (Table 1, Figure 1B), suggesting that dosage of BzpN affects proliferation. *bzpN*⁻ cells also proliferated faster than wild-type cells when grown on bacterial lawns, and this rapid proliferation was rescued by the expression of BzpN-GFP (Figure 1C). Together, these results indicate that BzpN functions to slow the proliferation of cells during exponential growth.

*bzpN*⁻ cells do not show a deficiency in AprA or CfaD accumulation

One explanation for the rapid proliferation of *bzpN*⁻ cells could be reduced extracellular AprA and/or CfaD accumulation due to aberrant expression, secretion, or degradation of these proteins. However, wild-type and *bzpN*⁻ cells showed similar levels of extracellular AprA and CfaD at both high and low cell densities (Figure 2 and data not shown). A 27 kD breakdown product of CfaD [19] appeared at higher levels in both *bzpN*⁻ and *bzpN*⁻/act15::bzpN-GFP conditioned media as compared to wild-type, although this difference likely does not significantly affect

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Figure 1. BzpN slows the proliferation of cells. (A) The *bzpN* transcript is not detected in *bzpN*⁻ cells during vegetative growth as assayed by RT-PCR. The mitochondrial ribosomal RNA IG7 serves as a control. (B) Log phase cells were diluted to 1 x 10⁶ cells/mL and grown in shaking culture. Values are means ± SEM (n=4) for all conditions. WT indicates wild type. All strains are in the AX4 background. (C) 1000 cells were spread on agar plates with bacteria. After 24 hours, the *Dictyostelium* cells were collected and counted. Values are means ± SEM (n=3). The differences in cell numbers between wild-type and *bzpN*⁻ and between *bzpN*⁻ and *bzpN*⁻/bzpN-GFP are significant (p<0.05, one-way ANOVA, Tukey’s test).

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proliferation, as suggested by the similar proliferation rates of wild-type and bzpN<sup>−</sup> / act15::bzpN-GFP cells. Silver staining of conditioned media samples showed similar protein levels, indicating equal loading and showing wild-type levels of extracellular protein accumulation in bzpN<sup>−</sup> cells. These results suggest that BzpN is not essential for normal extracellular accumulation of AprA and CfaD, and that the fast proliferation of bzpN<sup>−</sup> cells is not due to a lack of extracellular AprA or CfaD.

**bzpN<sup>−</sup>** cells show aberrant responses to AprA and CfaD

If BzpN is a component of an AprA/CfaD signal transduction pathway, bzpN<sup>−</sup> cells could be insensitive to the proliferation-inhibiting effects of AprA or CfaD. To test this possibility, we incubated wild-type, bzpN<sup>−</sup>, or bzpN<sup>−</sup> / act15::bzpN-GFP cells at low density with rAprA or rCfaD and determined the ability of these proteins to inhibit proliferation. As previously observed, rAprA and rCfaD inhibited the proliferation of wild-type cells [21,22] (Figure 3). However, rAprA had no significant effect on the proliferation of bzpN<sup>−</sup> cells, whereas rCfaD significantly increased bzpN<sup>−</sup> cell proliferation. In contrast, both rAprA and rCfaD inhibited the proliferation of bzpN<sup>−</sup> / act15::bzpN-GFP cells similar to wild-type. The increased proliferation of bzpN<sup>−</sup> cells in response to rCfaD may indicate the existence of a proliferation-promoting branch of the CfaD signal transduction pathway that serves to attenuate the proliferation-inhibiting effects of CfaD and that this proliferation-promoting pathway remains active in bzpN<sup>−</sup> cells. These results indicate that BzpN is necessary for the proliferation-inhibiting activity of AprA and CfaD and suggest that BzpN may function downstream of AprA and CfaD in a signal transduction pathway.

**bzpN<sup>−</sup>** cells secrete AprA and CfaD

Cells were grown to 12 ± 1 x 10<sup>6</sup> cells/mL in shaking culture and conditioned medium was collected and assayed by Western blot with anti-AprA or anti-CfaD antibodies. Asterisk indicates a 27 kDa breakdown product of CfaD. The intensity of this 27 kDa band and the light band at 50 kDa were variable between experiment replicates. Silver staining of conditioned media samples was done as a loading control. Numbers at the left indicate molecular mass in kDa. Data are representative of three independent experiments.

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**Table 1.** The effect of BzpN on the doubling time and stationary density of cells.

| Genotype                          | Doubling time, hours | Maximum observed cell density, 10<sup>6</sup> cells/mL |
|----------------------------------|-----------------------|--------------------------------------------------------|
| Wild type                        | 12.1±0.4              | 22.6±1.8                                               |
| bzpN<sup>−</sup>                  | 10.5±0.2*             | 25.2±1.3                                               |
| bzpN<sup>−</sup> / act15::bzpN-GFP | 13.7±0.9              | 25.0±2.5                                               |
| act15::bzpN-GFP                  | 14.0±0.4              | 14.4±0.6*                                              |

Doubling times and stationary densities were calculated for the proliferation curves in Figure 1. Values are mean ± SEM from four or more independent experiments. *indicates that the difference between the value and the wild-type value is significant with p<0.05 (one-way ANOVA, Tukey’s test).

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Figure 2. **bzpN<sup>−</sup>** cells secrete AprA and CfaD. Cells were grown to 12 ± 1 x 10<sup>6</sup> cells/mL in shaking culture and conditioned medium was collected and assayed by Western blot with anti-AprA or anti-CfaD antibodies. Asterisk indicates a 27 kDa breakdown product of CfaD. The intensity of this 27 kDa band and the light band at 50 kDa were variable between experiment replicates. Silver staining of conditioned media samples was done as a loading control. Numbers at the left indicate molecular mass in kDa. Data are representative of three independent experiments.

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protein accumulation as a function of time by dividing the observed mass and protein measurements by the measured doubling times, yielding the accumulation of mass or protein per hour. Mass and protein accumulation per cell was greater in AX4 than we have seen in AX2, though mass and protein accumulation per nucleus for AX4 are similar to what we have seen for AX2 [22].

bzpN2 cells accumulated mass, protein, and nuclei more rapidly than wild-type or bzpN2/act15::bzpN-GFP cells (Table 3). However, differences in the accumulation of mass or protein on a per nucleus basis were not significant between wild-type and bzpN2 cells (Table 3). These results suggest that BzpN, like AprA and CfaD, regulates proliferation but does not significantly regulate growth on a per nucleus basis.

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

Overexpression of BzpN in the wild-type background results in slow proliferation and a low stationary phase density (Figure 1B). To test whether AprA, CfaD, or QkgA are necessary for proliferation inhibition by BzpN, we expressed BzpN-GFP in cells lacking these proteins, confirmed expression by fluorescence microscopy, and examined the proliferation of these strains in parallel with the parental strains. As our aprA2, cfaD2, and qkgA2 cells are in the AX2 genetic background, we expressed BzpN-GFP in AX2 cells as a control, and saw a similar reduction in maximum cell density as in Figure 1B, where the AX4 strain was used (Figure 4). Whereas expression of BzpN-GFP in wild-type cells resulted in significantly lower cell densities during proliferation as compared to untransformed wild-type cells, expression of BzpN-GFP in aprA2, cfaD2, or qkgA2 cells resulted in no significant decrease in cell density during proliferation as compared to untransformed parental cells (Figure 4). These results suggest that AprA, CfaD, and QkgA are essential for the proliferation-inhibiting activity of BzpN.

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

aprA− and cfaD− cells yield fewer viable, detergent-resistant spores than wild type following multicellular development, indicating that AprA and CfaD promote the development of viable spores [18,19]. We tested the effect of BzpN on spore development by allowing wild-type, bzpN−, and bzpN−/act15::bzpN-GFP cells to develop, collecting and counting spores, and plating detergent-treated spores on lawns of bacteria. For all genotypes, the number of spores collected was higher than the number of cells allowed to develop (Table 4). This is a higher spore yield than we have previously observed, and may be due to the high percentage of multinuclear cells in the AX4 background, or a
round of cell proliferation during development, as has been previously observed [37]. Differences between any two genotypes for either the number of spores collected or the number of viable detergent-treated spores were not significant (Table 4). These results suggest that BzpN does not significantly affect spore viability and that AprA and CfaD affect spore development by a mechanism independent of BzpN.

Despite the fact that aprA<sup>2</sup> and cfaD<sup>2</sup> cells proliferate rapidly, colonies of these cells on a lawn of bacteria expand less rapidly than wild-type colonies [22], indicating that AprA and CfaD may function to facilitate the dispersal of groups of cells. To determine whether bzpN<sup>2</sup> cells expand slowly as colonies on a lawn of bacteria, we plated serial dilutions of wild-type and bzpN<sup>2</sup> cells mixed with <i>K. aerogenes</i> bacteria on SM/5 plates and measured the diameter of well-spaced plaques daily. No significant differences were observed between the sizes of wild-type and bzpN<sup>2</sup> plaques (data not shown), suggesting that AprA and CfaD facilitate the expansion of colonies in a manner independent of BzpN.

Conditioned media from wild type, aprA<sup>−</sup>, or cfaD<sup>−</sup> cells induce nuclear localization of BzpN-GFP

Basic leucine zipper transcription factors are known to translocate to the nucleus in response to extracellular signals in <i>Dictyostelium</i> [27]. To test whether AprA, CfaD, or conditioned media from high density cells are sufficient for nuclear translocation of BzpN, we added rAprA, rCfaD, both proteins, or conditioned medium from high density cells to low density cells expressing BzpN-GFP and examined localization of the BzpN-GFP fusion protein. The results are shown in Figure 4.

### Table 3. The effect of BzpN on the mass and protein accumulation of cells.

| Genotype               | Mass (mg) | Protein (µg) | Nuclei, x10<sup>6</sup> | Mass (mg) | Protein (µg) |
|------------------------|-----------|--------------|-------------------------|-----------|--------------|
| Wild type              | 0.74±0.03 | 42±2         | 14±1                    | 0.44±0.02 | 25±2         |
| bzpN<sup>+</sup>        | 1.02±0.02*| 59±2<sup>*</sup> | 19±1<sup>*</sup>        | 0.51±0.02 | 30±2         |
| bzpN<sup>−</sup>/actin15::bzpN-GFP | 0.66±0.04 | 36±2         | 12±1                    | 0.39±0.03 | 21±2         |

Mass and protein values from Table 2 were divided by the observed doubling time of the respective genotype. Doubling times were calculated as described in Materials and Methods. Values are the mean ± SEM from three or more independent experiments. *indicates that the difference between the value and the wild-type value is significant with p<0.05 (one-way ANOVA, Tukey’s test).

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Figure 4. AprA, CfaD, and QkgA are required for inhibition of proliferation by BzpN. Proliferation curves were done as described in Figure 1. Values are mean ± SEM, n=3. Expression of BzpN-GFP in wild-type cells resulted in a significant decrease in cell density on days 5 through 8 with p<0.05, whereas expression of BzpN-GFP in aprA<sup>−</sup>, cfaD<sup>−</sup>, or qkgA<sup>−</sup> cells did not cause a significant reduction in cell density at any measured timepoint (t-test). WT indicates wild type. All strains are in the AX2 background. Data for strains expressing BzpN-GFP are the average of two independent transformant clones.

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GFP fusion protein. In low-density cells, the fusion protein showed a punctate localization (Figure 5) that did not co-localize with either mitochondria or lysosomes (data not shown). The addition of AprA, CfaD, or both proteins resulted in no change in BzpN-GFP localization (data not shown). However, the addition of conditioned medium from high-density wild-type, aprA<sup>−</sup>, or cfaD<sup>−</sup> cells resulted in a nuclear localization of BzpN-GFP as judged by colocalization with DAPI staining (Figure 5). These results suggest that a factor present in conditioned media from high-density cells that is not AprA or CfaD mediates localization of BzpN to the nucleus.

BzpN prevents proliferation of cells in the presence of conditioned media from high-density cell cultures

Conditioned media from cells at stationary density prevents proliferation, and this inhibition may depend on an unidentified small molecule present in conditioned media [38]. As BzpN negatively regulates proliferation and BzpN-GFP localizes at the nucleus in response to conditioned media from high-density cells,
we tested whether Bzp\(N\) was necessary for this inhibition of proliferation. When conditioned media from stationary cells was added to wild-type cells at low cell density, we saw no increase in cell density after 24 hours (Figure 6). However, under the same conditions, \(bzpN^-\) cells showed a statistically significant increase in cell density, and this increase was not observed in \(bzpN^-/act15://bzpN-GFP\) cells. Cells resuspended in fresh media in parallel showed proliferation like we have previously observed for each genotype (Figure 1B, Table 1), indicating the viability of cultures inoculated into conditioned media (data not shown). These results indicate that a factor or factors present in stationary conditioned medium prevents cell proliferation and that Bzp\(N\) is necessary for the full activity of this factor.

**Discussion**

Apr\(A\) and Cfa\(D\) are autocrine signals that inhibit proliferation, though little is known regarding their mechanism of action. We provide evidence here showing that the putative transcription factor Bzp\(N\) is necessary for some of the responses to Apr\(A\) and Cfa\(D\). \(bzpN^-\) cells proliferate rapidly during exponential growth, as would be predicted for cells lacking downstream effectors of Apr\(A\) or Cfa\(D\). However, in contrast to apr\(A^-\) or cfa\(D^-\) cells, \(bzpN^-\) cells do not proliferate to a higher stationary density than wild-type cells. This suggests that Apr\(A\) and Cfa\(D\) may signal through a branched pathway, of which one branch functions to inhibit proliferation at lower densities and the other at higher densities. The Bzp\(N\)-independent branch may not be active in low-density cells, which would explain the inability of high-density conditioned media to arrest the proliferation of low-density \(bzpN^-\) cells (Figure 6). This model would predict the existence of mutants that proliferate like wild type cells at lower densities, but reach higher stationary densities than wild type. Mutants that show this phenotype have in fact been characterized [39].

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

Intriguingly, we saw that, although rApr\(A\) and rCfa\(D\) inhibited the proliferation of wild-type cells, rApr\(A\) and rCfa\(D\) increased the proliferation of \(bzpN^-\) cells, though only the increase in response to rCfa\(D\) was statistically significant. This result suggests the existence of a proliferation-promoting branch of the Cfa\(D\) signal transduction pathway that serves to attenuate the effect of the pathway on proliferation and that Bzp\(N\) is not a component of this branch. In \(bzpN^-\) cells, the proliferation-promoting element of the pathway appears to still be active, thereby resulting in an increase of proliferation in response to Apr\(A\) and Cfa\(D\).

We observed that Bzp\(N\)-GFP fusion proteins showed a punctate intracellular localization at low cell density, and addition of conditioned media from high-density cells resulted in a nuclear localization of Bzp\(N\)-GFP, indicating that an extracellular signal present at high cell density induces a localization of Bzp\(N\) at the nucleus. However, this localization occurred in response to conditioned media from either apr\(A^-\) or cfa\(D^-\) cells, and did not occur in response to rApr\(A\), rCfa\(D\), or both proteins. These results suggest that an extracellular signal that is not Apr\(A\) or Cfa\(D\) is required for the nuclear translocation of Bzp\(N\). Apr\(A\) and Cfa\(D\) may activate Bzp\(N\) by a mechanism independent of translocation, such as an activating phosphorylation that does not regulate subcellular localization, as is the case for the Arabidopsis bZIP transcription factor TRAB1 in response to the hormone abscisic acid [40]. Alternatively, a low basal level of nuclear Bzp\(N\) may be permissive in regards to Apr\(A\) and Cfa\(D\) signaling, perhaps by allowing the expression of a component of the Apr\(A\)/Cfa\(D\) signal transduction pathway. In this model Bzp\(N\) could be activated independently of Apr\(A\) or Cfa\(D\), causing the high level of nuclear localization, but still could be necessary for Apr\(A\) and Cfa\(D\) function.

It is unclear why Bzp\(N\) has been selected for during the evolution of Dicyostelium, apr\(A^-\) and cfa\(D^-\) cells show defects in spore generation [18,19] and in the expansion of colonies on solid substrates [22], correlating the loss of these genes with a lack of fitness under some circumstances. However, \(bzpN^-\) cells do not share these phenotypes, and thus Bzp\(N\) likely functions to increase fitness in some other context. Intriguingly, expression of Bzp\(N\) increases approximately 15-fold during the transition from growth to development [41], suggesting some role for Bzp\(N\) during the developmental stage. Bzp\(N\) may play a role in mitigating cellular stress, as cells lacking Bzp\(N\) show aberrant proliferation under conditions of nitrosative or thermal stress (see Supporting Information S1, Figure S1, and Figure S2). Alternatively, Bzp\(N\) may be functionally redundant with another protein affecting spore development or colony expansion, with the redundancy providing increased robustness.

Together, our data indicate that Bzp\(N\) is necessary for the inhibition of proliferation by the autocrine signals Apr\(A\) and Cfa\(D\),
but that AprA and CfaD function in other processes that do not require BzpN. An important but unanswered question is how the putative transcription factor activity of BzpN might be altering gene expression to regulate cell proliferation. The tractability of Dictyostelium genetics may be useful in identifying potentially conserved genes that are upregulated or downregulated in response to BzpN activity that function to regulate proliferation, which may reveal novel approaches to reduce the proliferation of cancers.

Supporting Information

Figure S1  Transcription of bzpN is induced under stress conditions.

A semi-quantitative RT-PCR was performed with RNA samples collected under various stress conditions: vegetative growth, starvation in KK2 buffer, incubation in HL5 with 500 μM SNP and incubation in HL5 at 30°C for 24 hours. IG7 was used as a control.

(TIF)

Figure S2  Proliferation of wild-type and bzpN− cells under stress conditions.

Exponentially growing cells were collected at 2 × 10^6 cells/ml and diluted to 5 × 10^5 cells/ml in HL5. (A) Cells were grown at 30°C to cause thermal stress. (B) 1 mM sodium nitroprusside (SNP) (Sigma) was added to the culture to introduce nitrosative stress and the cells were incubated at 32°C. Cells under all conditions were monitored by counting with hemocytometer over a week or until complete cell lysis was observed. Values are means ± SEM (n = 3). For proliferation at 30°C, differences in cell densities from days 2–7 are significant (p < 0.001, t-test). For proliferation in the presence of SNP, differences in densities are significant at the 140-hour timepoint (p < 0.05, one-tailed t-test).

(TIF)

Supporting Information S1  Supplement: Other activities of bzpN.

(DOC)

Author Contributions

Conceived and designed the experiments: JEP RHG EH GS. Performed the experiments: JEP EH. Analyzed the data: JEP RHG. Contributed reagents/materials/analysis tools: EH GS. Wrote the paper: JEP.

References

1. Kango-Singh M, Singh A (2009) Regulation of organ size: insights from the Drosophila Hippo signaling pathway. Dev Dyn 238: 1627–1637.
2. Gomer RH (2001) Not being the wrong size. Nat Rev Mol Cell Biol 2: 48–54.
3. Bulloch WS, Hewett CL, Laurence EB (1964) The Epithelial Chalone: A Preliminary Attempt at Isolation. Exp Cell Res 36: 192–200.
4. McPherson AC, Lavelle AM, Lee SJ (1995) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 378: 83–90.
5. Voadden MJ (1968) A chalone in the rabbit lens? Exp Eye Res 7: 326–331.
6. Garcia-Giralt E, Lasalvia E, Florentin I, Mathe G (1970) Evidence for a lymphocytic chalone. Rev Eur Etud Clin Biol 15: 1012–1015.
7. Houck JC, Weil RL, Sharma VK (1972) Evidence for a fibroblast chalone. Nat New Biol 248: 210–211.
8. Guba M, Cermatano G, Koehl G, Geissler EK, Jauch KW, et al. (2001) A primary tumor promotes dormancy of solitary tumor cells before inhibiting angiogenesis. Cancer Res 61: 5575–5579.
9. Peeters CF, de Waal RM, Wobbes T, Ruers TJ, et al. (2001) Mastocytic dormancy imposed by the primary tumor: does it exist in humans? Ann Surg Oncol 15: 3308–3315.
10. Demichelis R (2001) Tumour dormancy: findings and hypotheses from clinical research on breast cancer. Semin Cancer Biol 11: 297–306.
11. Brock DA, Gomer RH (1999) A cell-counting factor regulating structure size in Dictyostelium. Development 126: 259–268.
12. Jin R, Yuen IS, Taphouse CR, Gomer RH (1992) A density-sensing factor controls development in Dictyostelium. Genes Dev 6: 390–400.
13. Gomer RH, Yuen IS, Firtel RA (1993) A secreted 80 kD protein mediates sensing of cell density and the onset of development in Dictyostelium. Development 112: 269–278.
14. Kessin RH (2001) Dictyostelium : evolution, cell biology, and the development of multicellularity. Cambridge, UK ; New York: Cambridge University Press. xiv, 294 p.
15. Bonner JT (2003) Evolution of development in the cellular slime molds. Development 130: 2291–2302.
16. Aubry L, Firtel R (1999) Integration of signaling networks that regulate development in Dictyostelium. Genes Dev 6: 390–400.
17. Faix J, Kreppel L, Shaulsky G, Schleicher M, Kimmel AR (2004) A rapid and efficient method to generate multiple gene disruptions in Dictyostelium discoideum using a single selectable marker and the Cre-loxP system. Nucleic Acids Res 32: e143.
18. Ahern KG, Howard PK, Firtel RA (1980) Identification of regions essential for extrachromosomal replication and maintenance of an endogenous plasmid in Dictyostelium perioides. Nucleic Acids Res 16: 6823–6837.
19. Veltman DM, Akar G, Bosgraaf L, Van Haastert PJ (2009) A new set of small, extrachromosomal expression vectors for Dictyostelium discoideum. Plasmid 61: 110–118.
20. Sykiotis GP, Boimann D (2010) Stress-activated cap'n'collar transcription factors in aging and human disease. Sci Signal 3: re3.
21. Su TT, O'Farrell PH (1998) Size control: cell proliferation does not equal growth. Curr Biol 8: R507–R509.
22. Chen G, Shaulsky G, Kuspa A (2004) Tissue-specific G1-phase cell-cycle arrest prior to terminal differentiation in Dictyostelium. Development 131: 2619–2630.
23. Aiba K, Shintani K, Soll DR (1974) Observations on the growth of Dictyostelium discoideum in axenic medium: evidence for an extracelular growth inhibitor synthesized by stationary phase cells. J Cell Biol 41: 681–690.
24. Bokdo PB, Francione L, Bandala-Sanchez E, Ahmed AU, Annesley SJ, et al. (2007) Diverse cytopathologies in mitochondrial disease are caused by AMP-activated protein kinase signaling. Mol Biol Cell 18: 1874–1886.
25. Kango-Singh M, Singal A (2009) Regulation of organ size: insights from the Drosophila Hippo signaling pathway. Dev Dyn 238: 1627–1637.
26. Bonner JT (2003) Evolution of development in the cellular slime molds. Development 130: 2291–2302.
27. Manstein DJ, Schuster HP, Morandini P, Hunt DM (1995) Cloning vectors for extrachromosomal expression vectors for Dictyostelium discoideum. Plasmid 61: 390–400.
28. Barob E, Taphouse CR, Gomer RH (1992) A density-sensing factor controls development in Dictyostelium. Genes Dev 6: 390–400.
29. Manstein DJ, Schuster HP, Morandini P, Hunt DM (1995) Cloning vectors for extrachromosomal expression vectors for Dictyostelium discoideum. Plasmid 61: 390–400.