Extracellular Polyphosphate Inhibits Proliferation in an Autocrine Negative Feedback Loop in Dictyostelium discoideum*

Patrick M. Suess and Richard H. Gomer

From the Department of Biology, Texas A&M University, College Station, Texas 77843-3474

Polyphosphate is a polymer of phosphate residues linked by high energy phosphoanhydride bonds. Despite being highly conserved throughout nature, its function is poorly understood. Here we show that Dictyostelium cells accumulate extracellular polyphosphate, and this acts to inhibit proliferation at high cell densities. In shaking culture, extracellular polyphosphate concentrations increase as cell density increases, and if the concentration of polyphosphate observed at the stationary phase is added to cells at mid-log, proliferation is halted. Adding an exopolyphosphatase to cell cultures or stationary phase conditioned medium decreases polyphosphate levels and abrogates the anti-proliferative effect. The cells show saturable binding of polyphosphate, suggesting the presence of a cell surface polyphosphate receptor. Extracellular polyphosphate accumulation is potentiated by decreased nutrient levels, potentially as a means to anticipate starvation. Loss of the Dictyostelium polyphosphate kinase DdPpk1 causes intracellular polyphosphate levels to become undetectable and negatively affects fitness, cytokinesis, and germination. However, cells lacking DdPpk1 accumulate ~50% normal levels of extracellular polyphosphate, suggesting an additional means of synthesis. We found that cells lacking inositol hexakisphosphate kinase, which is responsible for the synthesis of the inositol pyrophosphates IP7 and IP8, reach abnormally high cell densities and show decreased extracellular polyphosphate levels. Two different enzymes thus appear to mediate the synthesis of Dictyostelium extracellular polyphosphate, which is used as a signal in an autocrine negative feedback loop to regulate cell proliferation.

Polyphosphate has the properties of the Dictyostelium stationary phase factor—a 2–3-kDa molecule (9, 10). We observed that this factor is resistant to proteinase K, DNase, and RNase treatment; does not partition into hydrophobic organic solvents; binds anion but not cation exchange resins; passes through a 3-kDa filter; and is retained by a 2-kDa filter (Table 1). This suggests that the stationary phase factor is a negatively charged ~2–3-kDa molecule.

Small polyphosphate molecules share the observed properties of the stationary phase factor. To determine whether polyphosphate could be acting as the stationary phase factor in Dictyostelium, we first tested whether cells accumulate extra-

* This work was supported by National Institutes of Health R01 GM102280. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: Dept. of Biology, Texas A&M University, ILSB 301 Old Main Dr., College Station, TX 77843-3474. Tel.: 979-458-5745; E-mail: rgomer@tamu.edu.

2 The abbreviations used are: CM, conditioned medium; polyP, polyphosphate.

Results

Polyphosphate Has the Properties of the Dictyostelium Stationary Phase Factor—Previous work indicated that the Dictyostelium stationary phase factor is a <10-kDa heat-resistant molecule (9, 10). We observed that this factor is resistant to proteinase K, DNase, and RNase treatment; does not partition into hydrophobic organic solvents; binds anion but not cation exchange resins; passes through a 3-kDa filter; and is retained by a 2-kDa filter (Table 1). This suggests that the stationary phase factor is a negatively charged ~2–3-kDa molecule.

Small polyphosphate molecules share the observed properties of the stationary phase factor. To determine whether polyphosphate could be acting as the stationary phase factor in Dictyostelium, we first tested whether cells accumulate extra-
TABLE 1
Effect of materials added to stationary phase CM on the ability of the medium to inhibit proliferation

| Material added to CM | CM activity |
|---------------------|-------------|
| Proteinase K        | +           |
| RNase               | +           |
| DNase               | +           |
| Hexane extraction   | Organic     |
| Organic             | +           |
| Aqueous             | +           |
| Ethyl acetate       | Organic     |
| Aqueous             | +           |
| Dichloromethane     | Organic     |
| Aqueous             | +           |
| Amberlite XAD-4     | −           |
| Bio-Rex 70 cation   | −           |
| Nucleic acid        | −           |
| Bio-Rex 70 cation   | 3-kDa cutoff spin filter |
| Flow-through        | +           |
| Bio-Rex 70 cation   | 2-kDa cutoff spin filter |
| Flow-through        | +           |

To determine whether nutrient levels altered polyphosphate accumulation, cells were cultured in concentrated SIH growth medium, a defined minimal medium, which caused increased proliferation and decreased polyphosphate accumulation per cell, whereas cells cultured in diluted growth medium or starvation buffer showed decreased proliferation and increased polyphosphate accumulation per cell (Fig. 2, A–C). Conditioned medium from starved cells also inhibited proliferation and contained ~40-mer polyphosphate (Fig. 2, D and E). Together, these results suggest that extracellular polyphosphate is acting as an inhibitor of proliferation to regulate population density in Dictyostelium, and its accumulation is potentiated by decreased nutrient availability.

The Proliferation-inhibiting Activity Is Reduced by Polyphosphate—To test the hypothesis that Dictyostelium cells secrete polyphosphate to inhibit their proliferation, we treated cell cultures and conditioned medium with ScPPX1. Daily additions of ScPPX1 to cultures caused cells to proliferate to higher densities compared with untreated cells (Fig. 3A). ScPPX1 treatment of conditioned media reduced extracellular polyphosphate to ~50 μM (Fig. 3B), a concentration where we observed little effect on proliferation (Fig. 1F), and this dramatically reduced the activity of the stationary phase factor in the conditioned medium (Fig. 3C). ScPPX1 treatment of conditioned starvation buffer also decreased polyphosphate levels (Fig. 3D).

Because phytic acid can also interact and fluoresce with DAPI at the same wavelengths as polyphosphate (24), conditioned medium was treated with the phytic acid-degrading enzymes phytase and DDP1 (25, 26). Phytase or DDP1 treatments had no effect on DAPI fluorescence of conditioned medium or on the activity of the stationary phase factor (Fig. 3, E and F). These results indicate that the polyphosphate in Dictyostelium stationary phase conditioned medium inhibits proliferation.

Polyphosphate Inhibits Cytokinesis to Cause an Increase in Cell Mass—Proliferation (an increase in cell number with time) and growth (an increase in cell mass with time) can be regulated separately (27). The stationary phase factor inhibits proliferation, yet compared with mid-log cells, stationary phase cells are larger (17). To determine whether polyphosphate inhibits proliferation but not growth, the cells were treated with polyphosphate or conditioned medium. Compared with controls, the cells treated with polyphosphate or conditioned medium showed increased cell mass and forward scatter in flow cytometer (Table 2). Cells in stationary phase showed an increase in multinucleate cells compared with mid-log cells (Table 3). To determine the effect of polyphosphate on cytokinesis, the cells were treated with polyphosphate for 18 h and then stained with DAPI. Polyphosphate caused a dramatic increase in multinucleate cells (Table 3). Aprotinin, secreted protein present in stationary conditioned medium, causes a decrease in the number of nuclei per cell (27), which may explain the increased number of nuclei per cell caused by polyphosphate compared with the number observed in stationary phase. These results show that the effects of polyphosphate on cell size match the observed properties of the stationary phase factor, appear to be due...
to polyphosphate inhibiting cytokinesis and suggest that polyphosphate has more of an inhibitory effect on cell proliferation than it does on cell growth or karyokinesis.

**Dictyostelium Binds Polyphosphate**—The presence of a cell surface polyphosphate receptor would require that cells show a class of saturable binding sites for polyphosphate. To determine whether polyphosphate binds to cells, the binding of biotinylated polyphosphate to wild type cells was measured. Long chain biotinylated polyphosphate showed saturable binding to wild type cells with a $K_d$ of 12 ± 3 μM, whereas medium chain biotinylated polyphosphate had a $K_d$ of 78 ± 32 μM (mean ± S.E., n = 4), suggesting that longer polymers have a higher binding affinity (Fig. 4A). The Hill coefficient was 1.0 ± 0.3 for both chain lengths, suggesting that there is no binding cooperativity, and F tests comparing models with multiple binding sites indicated a single class of binding site. The $B_{max}$ of medium chain polyphosphate was 1.6 ± 1.1 × 10^6 binding sites/cell. Although this number is high compared with known Dictyostelium cell surface receptors (28–30), there are many examples of numbers of cell surface binding sites in this range and higher (31–35). The binding appeared to equilibrate within 1 min (Fig. 4B). The binding of medium chain biotinylated polyphosphate could be competed with both conditioned medium and unlabeled polyphosphate (Fig. 4, C and D). Using the equation of Cheng and Prusoff (36), unlabeled polyphosphate showed a $K_i$ of 87.5 ± 7.2 μM. To check that the binding of polyphosphate to cells was not simply due to anionic interactions, binding was competed using sodium sulfate, another anionic molecule (Fig. 4E). Sodium sulfate was not able to compete with polyphosphate for binding, suggesting that polyphosphate binding is not simply an anionic interaction with the cell membrane. Together, these results indicate that polyphosphate shows saturatable binding to cells, and the binding can be competed with unlabeled polyphosphate and stationary phase conditioned media.

**FIGURE 1.** Wild type cells accumulate increasing amounts of extracellular polyphosphate as cell density increases. A and B, cells were cultured, and the polyphosphate (polyP) concentration in the conditioned medium was measured. C, polyP from stationary phase conditioned medium was resolved on a 25% polyacrylamide gel and stained with toluidine blue (representative image of three gels). D and F, cells were cultured with 150 μM polyP (D) or the indicated concentration of polyP (F) and density (D), and the percentage of inhibition after 24 h (F) was determined. E, wild type cells were cultured in the presence or absence of 150 μM polyP for 18 h, washed twice in HLS, resuspended in fresh HLS, and counted daily. G, wild type cells were cultured in the presence or absence of triphosphate, pyrophosphate, or sodium phosphate and counted daily. All values are means ± S.E., n = 4. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (one-way analysis of variance).
Inositol Hexakisphosphate Kinase Regulate Extracellular Polyphosphate Accumulation—Inositol hexakisphosphate kinase (IP6K) uses IP6 as a substrate to generate the inositol pyrophosphates IP7 and IP8 (37, 38). Yeast lacking the IP6K Kcs1 have no observable polyphosphate accumulation (14, 16). Mice lacking IP6K have reduced polyphosphate levels in platelets (15). To test the hypothesis that IP6K also regulates polyphosphate levels in Dictyostelium, we measured extracellular polyphosphate in cells lacking the IP6K I6kA, as well as cells lacking DdPpk1 (21, 39). The cells lacking either I6kA or DdPpk1 proliferated to abnormally high cell densities and had reduced extracellular polyphosphate accumulation (Fig. 5, A–D). PAGE indicated that both mutants accumulate less extracellular polyphosphate than wild type (Fig. 5E). In agreement with the abnormally low levels of extracellular polyphosphate in cultures of i6kA− and ppk1− cells, conditioned medium from i6kA− and ppk1− cells harvested at densities in which wild type cells enter stationary (20–25 × 10⁶ cells/ml) or at maximum densities (30–40 × 10⁶ cells/ml) contained abnormally low levels of inhibitory activity (Fig. 5F). The abnormally high maximum density and low polyphosphate accumulation phenotypes exhibited by i6kA− cells were rescued by expressing the I6kA cDNA under the control of the actin 15 promoter (Fig. 5, C, D, and G). Polyphosphate supplementation effectively rescued the i6kA− proliferation defect, suggesting that this defect is due to reduced stationary phase factor production as opposed to reduced sensitivity to the factor itself (Fig. 5G). The proliferation of ppk1− cells could also be inhibited by exogenous polyphosphate, although these cells showed a roughly 2-fold reduction in sensitivity (Fig. 5H). Consistent with these observations, both I6kA and DdPpk1 are up-regulated upon transitioning from vegetative growth to starvation as reported by the DictyExpress. Together, these results show roles for both I6kA and DdPpk1 in regulating extracellular polyphosphate accumulation in Dictyostelium, as well as a conserved role for inositol hexakisphosphate kinase in polyphosphate accumulation in multiple eukaryotic models.

To determine whether intracellular polyphosphate is also regulated by both DdPpk1 and I6kA, intracellular polyphosphate was measured in wild type cells and cells lacking I6kA. As previously reported, intracellular polyphosphate levels are highest as cells enter the stationary phase (19), showing a roughly 2-fold increase from mid-log to stationary (Fig. 5I). Cells lacking I6kA had normal amounts of intracellular polyphosphate at low cell densities but did not show the robust increase in intracellular polyphosphate production at high cell densities. Although DdPpk1 is essential for intracellular polyphosphate production at all cell densities, I6kA appears to
have a role in regulating intracellular polyphosphate levels at high cell densities.

**Mutant Proliferation Phenotypes Correlate with Extracellular Polyphosphate Accumulation**—We previously identified transformants that proliferate to abnormally high or low stationary phase or maximum cell densities (27, 40–42). In addition to cells lacking I6kA and DdPpk1, cells lacking the G proteins G/H9252, G/H9251, and G/H92519, the secreted proteins AprA and CfaD and the kinase PakD all proliferate to abnormally high cell densities (Fig. 6A). For instance, AprA and CfaD slow but do not stop cell proliferation and thus have some stationary phase factor-like properties (27, 40). All of these strains had abnormally low levels of extracellular polyphosphate/1 × 10⁶ cells at their respective maximum densities (Fig. 6B). The cells lacking phos-
phospholipase D, a homolog of human PLD1, and the cells overexpressing AprA proliferate to abnormally low stationary cell densities (Fig. 6A) and had abnormally high levels of extracellular polyphosphate (Fig. 6B). These results suggest that for some transformants, maximal cell density shows an inverse correlation with extracellular polyphosphate level.

Discussion

In this report, we found that the extracellular proliferation inhibitor originally identified by Yarger, Soll, and co-workers (17, 18), which causes Dictyostelium cells to stop proliferating at stationary phase (17, 18), is 9-mer polyphosphate. When extracellular polyphosphate is depleted by treatment with exopolyphosphatase, the cells grow to a higher density. At first glance, having polyphosphate inhibit proliferation would thus seem to be disadvantageous to the cells. However, we found that polyphosphate has more of an inhibitory effect on proliferation than on growth, resulting in high density cells stopping proliferation when they have large amounts of stored nutrients. This then suggests that when a population of cells starves, having somewhat fewer cells with more stored nutrients per cell is more advantageous than having more cells with less stored nutrients per cell. We envision that polyphosphate thus allows proliferating cells to anticipate the inevitable situation where a high density of cells overgrows its food supply and starves. Decreased nutrient levels potentiate extracellular polyphosphate accumulation, potentially allowing the cells to more accurately predict when they will starve.

When starved, Dictyostelium cells lacking DdPpk1, and therefore intracellular polyphosphate, form smaller fruiting bodies and have defective spore germination (21). We found that extracellular polyphosphate may help cells anticipate and prepare for starvation by stopping proliferation when they have relatively high levels of stored nutrients. Polyphosphate accumulation in Dictyostelium thus potentiates survival in stressful conditions as it does in many prokaryotes (1, 5).

Although polyphosphate present in starved conditioned medium can be degraded to undetectable levels, some of the polyphosphate present in stationary phase conditioned medium appears to be resistant to yeast exopolyphosphatase. Kornberg and co-workers (1) observed that preparations of polyphosphate from biological sources also show resistance to exopolyphosphatase activity, presumably through a terminal end modification, which can confer resistance to exopolyphosphatase (43). Some of the stationary phase extracellular polyphosphate may thus also have covalent modifications.

Although the saturable binding of polyphosphate to cells suggests the existence of a cell surface polyphosphate receptor, it does not prove the existence. If the observed binding is to a receptor that causes cells to stop proliferating, with the observed first order binding kinetics, using percentage bound = [ligand]/([ligand] + Ks), at 150 μM polyphosphate and the observed Ks of unlabeled polyphosphate (87.5 μM), the receptor stops proliferation when ~63% of the receptors are occupied.

FIGURE 4. Polyphosphate binds wild type cells. A, mid-log wild type cells were incubated with the indicated amounts of biotinylated polyP and a streptavidin-conjugated fluorophore. The cells were washed, and fluorescence was measured using a flow cytometer. B, cells were incubated with 200 μM medium chain biotinylated polyP for the indicated times, and binding was measured as in A. C, D, and E, competition assays were performed by incubating wild type cells with 200 μM medium chain biotinylated polyP, streptavidin-conjugated fluorophore, and either stationary phase conditioned medium (C), unlabeled polyP (D), or sodium sulfate (E) binding was measured as in A. Concentrations of unlabeled polyP (D) are shown as whole polyphosphate molecules as opposed to phosphate monomers. The values are means ± S.E., n ≥ 4.
Cells lacking DdPpk1 have undetectable levels of intracellular polyphosphate (21), whereas we observed that these same cells have reduced but detectable levels of extracellular polyphosphate. Polyphosphate accumulation in yeast and mice involves inositol hexakisphosphate kinase, and we observed that this enzyme also contributes to the accumulation of extracellular and intracellular polyphosphate at high cell densities in Dictyostelium. Thus, for unknown reasons, Dictyostelium cells appear to use at least two different enzymes to generate polyphosphate.
The secreted proteins AprA and CfaD; the G protein subunit Gg1, Gg9 (which mediates AprA signaling (41)), and Gb; and the putative kinases PakD and PldB all modulate extracellular polyphosphate accumulation and maximal cell density. A reasonable assumption is that these signals and pathways are involved in sensing cell density, nutrient status, or other conditions that to some extent help cells accumulate enough polyphosphate to anticipate starvation and stop proliferating. Little is known about how the size or composition of a tissue is regulated. One possibility is that cells of a specific type secrete a factor called a chalone. Chalones are factors that inhibit the proliferation of the cells that secrete them (44, 45). As the number of cells secreting the chalone increases, the extracellular concentration of the chalone increases, and at some combination of cell number and chalone concentration, cell proliferation stops (45). Despite considerable evidence for their existence in mammalian tissues such as epidermis, intestine, spleen, liver, and kidney, for many chalones, attempts to identify them as either organic molecules or proteins have failed (45–51). Extracellular polyphosphate appears to be acting as a chalone in *Dictyostelium*. Together, these results suggest a new role for polyphosphate, as well as the intriguing possibility that chalones in higher eukaryotes might be compounds that are neither conventional organic molecules nor polypeptides.

**Experimental Procedures**

**Reagents and Materials**—HL5 and SIH growth media were from Formedica (New Brunswick, NJ) and Merck. Phytic acid and high density nickel beads were from GoldBio (St. Louis, MO). Phytase, hexane, ethyl acetate, dichloromethane, sodium triopolyphosphate, sodium pyrophosphate, acetylated phenolchloform, and Amberlite XAD-4 were from Sigma. DDP1 from MyBioSource (San Diego, CA). Biotinylated polyphosphate was from KeraFast Inc. (Boston, MA). Streptavidin-conjugated Alexa Fluor 647 was from Invitrogen. Bystacidin and G418 were from CalBioChem (Boston, MA). Vectors and mutant cell lines were obtained from dictyBase. Size exclusion spin filters were from Sartorius (Bohemia, NY) (2 kDa) and Aviva Biosciences (San Diego, CA) (10 and 3 kDa). Proteinase K, RNase, DNase, and restriction enzymes were from New England Biolabs (Ipswich, MA). All cation and anion exchange beads were from Bio-Rad.

**Cell Culture and Proliferation Curves**—The cells used were *Dictyostelium* wild type Ax2, aprA− (DB60T3–8), cfaD− (DB27C–1), gB− (DB0236531), ga1− (DB02306088), ga9− (DB0236109), i6kA− (DB0236426), ppp1− (DB0350686), pakD− (DB0350281), pldB− (DB0236796), and *AprA*ΔE (DB0233510). Mutant genotypes were verified by PCR and kept under constant selection. The cells were grown in shaking culture using HL5 nutrient rich growth medium (10–12-h doubling time) or the synthetic medium SIH (−22–26-h doubling time). Proliferation curves used mid-log cells diluted to 5 × 10^5 cells/ml, and density was measured every 24 h using a hemocytometer. Conditioned medium was harvested by centrifugation of cells at 800 × g for 10 min at 4 °C, and the supernatant was saved. The supernatant was clarified by centrifugation at 9000 × g for 10 min at 4 °C, and the resultant supernatant was filter sterilized using 0.22-μm filters (Genssee Scientific, San Diego, CA) and stored at 4 °C. The cells were starved in PBM (27) for 5 h at 5 × 10^6 cells/ml, and conditioned medium was collected. Proliferation inhibition by conditioned medium was assessed by culturing cells in 50% conditioned medium and 50% growth medium. The percentage of inhibition was determined by treating control cells as 100% proliferation, 0% inhibition and wild type conditioned medium as 100% inhibition, 0% proliferation. The cell mass and nuclei content were measured as previously described (27). Forward scatter (in arbitrary units) was measured on a BD Accuri C6 (BD Biosciences, San Jose, CA) flow cytometer.

**Treatments of Conditioned Media**—Proteinase K, RNase, and DNase were added to SIH conditioned medium to 0.1 mg/ml at 37 °C for 18 h, and the enzyme was then removed using a 10-kDa cutoff spin filter. Organic extractions were done by adding an equal volume of solvent, vortexing for 10 s every 2–3 min for 20 min, phase separation by centrifugation at 200 × g for 5 min, and then lyophilization of the organic and aqueous phases. The organic phase was resuspended in SIH, and the aqueous phase was resuspended in water. Bead treatments involved washing beads following the manufacturer’s protocol, adding a 30% volume of bead slurry to conditioned medium, rotating gently for 1 h, letting the beads settle out, and then clarification of the supernatant at 200 × g for 5 min.

**Polyphosphate Measurements**—Polyphosphate levels in conditioned medium were assessed by incubating samples with DAPI and measuring fluorescence at 550 nm when excited at 415 nm as previously described (22) with the following modifications. Conditioned medium samples were generated in the synthetic media SIH to reduce the amount of background fluorescence from HL5 medium and were clarified using a 10-kDa spin filter (except for starved CM), and the filtrate was saved. Conditioned medium was then incubated with 25 μg/ml DAPI for 5 min, and the fluorescence was measured. Polyphosphate concentrations, in terms of phosphate monomers, were determined using polyphosphate standards generously provided by Dr. Toshikazu Shiba (RegeneTiss Inc.).

Intracellular polyphosphate levels were assessed by resuspending cell pellets in LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8, 0.5% SDS). One volume of acidic phenolchloroform (pH 4) was added, and samples were vortexed for 5 min at 4 °C. The samples were then spun at 17,000 × g for 5 min at 4 °C. Two volumes of chloroform were added to the resulting aqueous phase, vortexed for 5 min at 4 °C, and then spun at 2000 × g for 5 min at 4 °C. The resulting aqueous phase was incubated with 2.5 volumes of 100% ethanol for 5 min at room temperature and then spun at 15,000 × g for 10 min at 4 °C. The pellet was resuspended in 100 μl of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8), containing 1 μg/ml of RNase A and incubated for 15 min at room temperature. 2.5 volumes of 100% ethanol were then added, and the mixture was incubated at −80 °C for 1–2 h and then spun at 15,000 × g for 10 min at 4 °C. The pellet was resuspended in TE. The samples were then assayed for polyphosphate content as described above using DAPI.

**PAGE Analysis**—Polyphosphate was resolved on polyacrylamide gels as previously described (38). Stationary phase and starved conditioned media were concentrated 15-fold using 2-kDa size exclusion filters. Polymer lengths were calculated by plotting the log of polymer lengths against the position of the band on a gel.
**Enzyme Treatments**—Plasmids for purifying yeast PPX1 were kindly provided by Dr. Michael Gray (52). Recombinant protein purification was performed as previously described (27). Treatments of conditioned media using 0.15 µg/ml recombinant ScPPX1 were done for 4 h at 37 °C, supplementing the medium with 5 mM MgCl₂. Phytase was added to 3 µg/ml (crude extract) for 4 h at pH 5.5 at 37 °C. pH was adjusted using 1 N HCl or 1 N NaOH followed by filter sterilization using 0.22-µm filters. DPD treatments at 0.15 µg/ml were done for 4 h at 37 °C in the presence of 5 mM MgCl₂. The reactions were passed through 10-kDa cutoff spin filters to remove enzyme and then assayed for polyphosphate content and chalone activity. For ScPPX1 treatment of cell cultures, mid-log cells were inoculated at 5 × 10⁶ cells/ml, and ScPPX1 or an equal volume of buffer was added daily.

**Binding Assay with Biotinylated Polyphosphate**—Binding of polyphosphate to wild type cells was determined using biotinylated polyphosphate. The cells at mid-log were washed twice with ice-cold PBM and then resuspended in ice-cold PBM. The samples were incubated with biotinylated compound (100 µM for competition assays) and streptavidin-conjugated Alexa Fluor 647 (1:200) on ice for 3 min, centrifuged at 200 × g for 3 min at 4 °C, and resuspended in ice-cold PBM, and fluorescence was measured using a flow cytometer. Conditioned medium was 10-kDa filtered to remove any large molecules or proteins.

The total number of binding sites/cell was calculated by incubating 10⁷ cells for 3 min on ice with 150 µM biotinylated polyphosphate in 200 µL. The samples were then centrifuged at 200 × g for 3 min at 4 °C, and the supernatant was harvested without disruption of the cell pellet. The supernatant was then incubated with mid-log cells as described above. Comparison of the supernatant binding and the binding of different concentrations of biotinylated polyphosphate to cells was used to determine the amount of biotinylated polyphosphate bound to the 10⁷ cells, and this was used to calculate the Bₘᵡₜ.

**Expression of i6kA in i6kA⁻ Cells**—To generate the i6kA⁻ / a15::i6kA cell line, the i6kA open reading frame was amplified by PCR from wild type vegetative cDNA with the primers 5'-C-GCGAACGCTTATGACATATTAGTTATAGTA and 5'-CCGGGATCTTTAGTTATGTATGACTGTAT with terminal HindIII and BamHI sites, respectively. This PCR product was cloned into pGEM-T vector (Promega). The i6kA open reading frame was isolated by HindIII and BamHI digestion and gel purification and then ligated into the HindIII and BamHI sites of pDXa-3C. Correct orientation of the open reading frame within the vector was confirmed by restriction mapping and sequencing. This vector was transformed into Dictyostelium cells lacking i6kA following (27), using 5 µg/ml G418 to select for cells containing the vector.

**Author Contributions**—P. M. S. designed, performed, and analyzed the experiments and wrote the paper. R. H. G. conceived and coordinated the study, acquired funding, and revised the final paper.

**Acknowledgments**—We thank Bethany Sump and Jacob Watson for assistance, Dr. Toshikazu Shiba of RegeneTiss Inc. for polyphosphate standards, Dr. Michael Gray for plasmids, and Thomas Livermore and the Saiardi Lab at University College London for advice on PAGE analysis of polyphosphate.
karyote, Dictyostelium discoideum, with a role in cytokinesis. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 16486–16491
21. Livermore, T. M., Chubb, J. R., and Sairardi, A. (2016) Developmental accumulation of inorganic polyphosphate affects germination and energetic metabolism in *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 996–1001
22. Aschar-Sobbi, R., Abramov, A. Y., Diao, C., Kargacin, M. E., Kargacin, G. J., French, R. J., and Pavlov, E. (2008) High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *J. Fluoresc.* **18**, 859–866
23. Wurst, H., and Kornberg, A. (1994) A soluble exopolyphosphatase of *Saccharomyces cerevisiae*: Purification and characterization. *J. Biol. Chem.* **269**, 10996–11001
24. Kolozsvari, B., Parisi, F., and Sairardi, A. (2014) Inositol phosphates induce DAPI fluorescence shift. *Biochem. J.* **460**, 377–385
25. Peers, F. G. (1953) The phytase of wheat. *Biochem. J.* **53**, 102–110
26. Lonetti, A., Szijgyarto, Z., Bosch, D., Loss, O., Azevedo, C., and Saiardi, A. (2014) Activation of *Dictyostelium discoideum* chemotaxis by gel electrophoresis. *PLoS One* **9**, e85533
27. Brock, D. A., and Gomer, R. H. (2005) A secreted factor represses cell proliferation. *BMC Biol.* **10**, 4
28. Peers, F. G. (1953) The phytase of wheat. *Biochem. J.* **53**, 102–110
29. Brock, D. A., and Gomer, R. H. (2005) A secreted factor represses cell proliferation in *Dictyostelium*. *Development* **132**, 4553–4562
30. Choe, J. M., Bakthavatsalam, D., Phillips, J. E., and Gomer, R. H. (2009) *Dictyostelium* cells bind a secreted autocrine factor that represses cell proliferation. *BMC Biol.* **10**, 4
31. Caterina, M. J., Hereld, D., and Devreotes, P. N. (1995) Occupancy of the *Dictyostelium* cAMP receptor, cAR1, induces a reduction in affinity which depends upon COOH-terminal serine residues. *J. Biol. Chem.* **270**, 4418–4423
32. Wurst, B., and Butz, U. (1980) Reversible binding of the chemoattractant folic acid to cells of *Dictyostelium discoideum*. *Eur. J. Biochem.* **109**, 613–618
33. Low, I. E., and Jardine, I. (1986) A novel cyclosporine binding assay. *J. Pharmacol. Exp. Ther.* **238**, 39–45
34. Müller, A., Rice, P. J., Ensley, H. E., Kargacin, M. E., Kargacin, G. J., French, R. J., and Pavlov, E. (2008) High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *J. Fluoresc.* **18**, 859–866
35. Caterina, M. J., Hereld, D., and Devreotes, P. N. (1995) Occupancy of the *Dictyostelium* cAMP receptor, cAR1, induces a reduction in affinity which depends upon COOH-terminal serine residues. *J. Biol. Chem.* **270**, 4418–4423
36. Britigan, B. E., Serody, J. S., and Cohen, M. S. (1994) The role of lactoferrin as an anti-inflammatory molecule. *Adv. Exp. Med. Biol.* **357**, 143–156
37. Soda, R., and Tavassoli, M. (1984) Liver endothelium and not hepatocytes or Kupfer cells have transferrin receptors. *Blood* **63**, 270–276
38. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108
39. Morrissey, J. H. (2013) One inositol ring to rule thrombosis. *Blood* **122**, 1331–1332
40. Metcalf, D. (1964) Restricted growth capacity of multiple spleen grafts. *Exp. Cell Res.* **2**, 52–59
41. Bakthavatsalam, D., Choe, J. M., Hanson, N. E., and Gomer, R. H. (2009) A *Dictyostelium* chalone uses G proteins to regulate proliferation. *BMC Biol.* **7**, 4
42. Phillips, J. E., and Gomer, R. H. (2014) The p21-activated kinase (PAK) family member PakD is required for chemorepulsion and proliferation inhibition by autocrine signals in *Dictyostelium discoideum*. *PLoS One* **9**, e96633
43. Choi, S. H., Collins, J. N., Smith, S. A., Davis-Harrison, R. L., Rienstra, C. M., and Morrissey, J. H. (2010) Phosphoramidate end labeling of inorganic polyphosphates: facile manipulation of polyphosphate for investigating and modulating its biological activities. *Biochemistry* **49**, 9935–9941
44. Bullough, W. S. (1965) Mitotic and functional homeostasis: a speculative review. *Cancer Res.* **25**, 1683–1727
45. Gomer, R. H. (2001) Not being the wrong size. *Nat. Rev. Mol. Cell Biol.* **2**, 48–54
46. Bolding, W. H., and Laurence, E. B. (1968) Extraction, purification and preliminary characterisation of the epidermal chalone: a tissue specific mitotic inhibitor obtained from vertebrate skin. *Eur. J. Biochem.* **5**, 191–198
47. Bullough, W. S., Hewett, C. L., and Laurence, E. B. (1964) The epidermal chalone: a preliminary attempt at isolation. *Exp. Cell Res.* **36**, 192–200
48. Metcalf, D. (1964) Restricted growth capacity of multiple spleen grafts. *Transplantation* **2**, 387–392
49. Michalopoulos, G. K., and DeFrances, M. (2005) Liver regeneration. *Adv. Biochem. Eng. Biotechnol.* **93**, 101–134
50. Saetren, H. (1956) A principle of auto-regulation of growth; production of organ specific mitose-inhibitors in kidney and liver. *Exp. Cell Res.* **11**, 229–232
51. Sassi, P., and Bergeron, M. (1977) Specific inhibition of cell proliferation in the mouse intestine by an aqueous extract of rabbit small intestine. *Cell Tissue Kinetics* **10**, 223–231
52. Gray, M. J., Wholey, W. Y., Wagner, N. O., Cremers, C. M., Mueller-Schickert, A., Hock, N. T., Krieger, A. G., Smith, E. M., Bender, R. A., Bardwell, J. C., and Jakob, U. (2014) Polyphosphate is a primordial chaperone. *Mol. Cell* **53**, 689–699