Developmental accumulation of inorganic polyphosphate affects germination and energetic metabolism in *Dictyostelium discoideum*

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Inorganic polyphosphate (polyP) is composed of linear chains of phosphate groups linked by high-energy phosphoanhydride bonds. However, this simple, ubiquitous molecule remains poorly understood. The use of nonstandardized analytical methods has contributed to this lack of clarity. By using improved polycrystalline gel electrophoresis we were able to visualize polyP extracted from *Dictyostelium discoideum*. We established that polyP is undetectable in cells lacking the polyphosphate kinase (DdPpk1). Generation of this *ppk1* null strain revealed that polyP is important for the general fitness of the amoebae with the mutant strain displaying a substantial growth defect. We discovered an unprecedented accumulation of polyP during the developmental program, with polyP increasing more than 100-fold. The failure of *ppk1* spores to accumulate polyP results in a germination defect. These phenotypes are underpinned by the ability of polyP to regulate basic energetic metabolism, demonstrated by a 2.5-fold decrease in the level of ATP in vegetative cells lacking the polyphosphate kinase (DdPpk1). Finally, the lack of polyP during the development of *ppk1* mutant cells is partially offset by an increase of both ATP and inositol polyphosphates, evidence for a model in which there is a functional interplay between inositol pyrophosphates, ATP, and polyP.

**Significance**

The most basic biological polymer is a linear chain of linked phosphate groups, simply called inorganic polyphosphate (polyP). By ablating polyP synthesis in the amoebae, *Dictyostelium discoideum*, we discovered that polyP regulates basic metabolism, general fitness, and spore germination. We also discovered a massive increase in the level of polyP during developmental progression. Interestingly this accumulation is linked to the accumulation of another family of phosphate-rich molecules, the inositol polyphosphates. Thus, by using a genetic model, we reveal a link between polyP, ATP production, and inositol polyphosphates.

The intact VTC complex is required for transport of polyP into the vacuole lumen (15), leading to the accumulation of polyP in the vacuole. Both exo- and endo polyphosphatases have also been identified in yeast (16, 17). Furthermore, the yeast model has revealed a metabolic link between polyP and the phosphate-rich inositol pyrophosphates. In yeast, the levels of inositol pyrophosphates closely correlate with the level of polyP, and yeast strains lacking inositol polyphosphates possess almost no polyP (17).

PolyP has also been documented in the social amoeba *Dictyostelium discoideum*. Its presence in amoebae was first recognized by biochemical extraction in the early 1970s (18). Subsequently, in the late 1980s, the use of 31P-NMR analysis revealed that both InoP6 and polyP are present in *Dictyostelium* spores (19). Besides yeast, the social amoeba is the only other eukaryote where the enzymology of polyP synthesis has been studied. Kornberg’s laboratory described two enzymes able to synthesize polyP in *D. discoideum*. The first, DdPPK1 (hereafter refer as Ppk1), was identified by homology to the bacterial polyphosphate kinase (PPK) sequence (20). Dictyostelids represent the only eukaryote class possessing a bacterial-like PPK, suggesting the gene was obtained by a horizontal gene transfer event (20). The second protein complex, named DdPPK2, was identified after biochemical purification of polyphosphate kinase activity and was found to comprise one actin-related protein and two actin-associated proteins (21). Whereas the in vitro biochemical properties of these two enzymes have been studied (20, 22), little is known about *D. discoideum* polyP metabolism and the contribution of these proteins to in vivo polyP synthesis, or the physiological function of polyP. Therefore, we have used a recently refined biochemical procedure to study polyP metabolism in *D. discoideum*. Our analysis failed to detect the presence of polyP in cells lacking the bacterial-like Ppk1. Importantly, we observed a dramatic increase of polyP during amoebae development.
with an accumulation in the spore ultimately affecting germination. These defects are due to the influence of polyP on the energetic status of the cell and inositol pyrophosphate metabolism.

**Results**

**D. discoideum Possesses polyP.** Several polyP detection methods lack specificity (23). Furthermore, quantification of polyP using traditional biochemical assays, such as the PPK assay (8) or measurement of free phosphate (Pi) after polyP hydrolysis, are greatly impaired by the inability to extract polyP without copurifying other phosphate rich molecules (see below). Therefore, the only approach to unambiguously establish the presence of polyP in cells is its visual detection once resolved by polyacrylamide gel electrophoresis (PAGE) (24). Furthermore, the ability of the phosphohydryde bonds of polyP to induce photobleaching of DAPI has improved the sensitivity of this method over traditional Toluidine staining (25).

We extracted polyP from vegetative growing *D. discoideum* AX2 cells using phenol/chloroform, which also extracts RNA and inositol pyrophosphates, and resolved 70 μg of RNA by 20% PAGE. DAPI staining revealed the negatively stained smear typical of polyP-DAPI photobleaching in cell extracts (Fig. 1). Our extraction procedure copurifies polyP and nucleic acids, mainly RNA; thus to be confident of the nature of the polyP negative smear, we subjected the extract to DNase and RNase treatment. Whereas RNase treatment removed the positive fluorescence visible at the top of the gel (Fig. 1), neither DNase nor RNase affected the negative staining. To test whether polyP was the constituent of this negative staining, we treated the samples with the endopolyphosphatase Ddp1 (17) and the exopolyphosphatase Ppx1 (16). This enzymatic treatment removed the negative smear generated by polyP standards as well as that present in cell extracts (Fig. 1). We further confirmed the smear to be due to polyP by treating with acid at high temperature (acid boiled), because phosphohydryde bonds are rapidly degraded by these conditions. These analyses demonstrated the presence of polyP in vegetative *D. discoideum*, not only from the standard laboratory strain AX2, but also in the natural isolate, NC4 (Fig. 1). By comparing the migration of *D. discoideum* polyP with polyP standards, we estimate that amoebae possess polyP with an average length of ~50 phosphate residues.

**Ppk1 the Only Enzyme Responsible for polyP Synthesis Control General Fitness and ATP Levels.** The characterization of two *D. discoideum* enzymes Ppk1 (20) and DdPPK2 (22) able to synthesize polyP in vitro suggested that both enzymes might contribute to the synthesis of polyP in vivo. DdPPK2 has been identified as an actin-related protein complex but the specific gene was not cloned. Given that the *D. discoideum* genome encodes 41 actins and actin-related proteins (26) it was impractical to systematically delete all of these genes. Because only one bacterial-like Ppk1 is present in the *Dictyostelium* genome, we deleted, by homologous recombination, 69% of the ORF of this gene (ID: DDB_G0293524) as confirmed by Southern blots (Fig. 2A and B). We referred to the Ppk1 null strain as ppk1. Two independent clones were generated and used in our experiments. The homology between the 69% aa of *E. coli* PPK1 and the 1.050 aa of *D. discoideum* Ppk1 is restricted to the C terminus; therefore, our knockout approach deleted >95% of the Ppk1 catalytic domain (Fig. 24). We first analyzed the general fitness of ppk1 cells by performing a growth assay in rich HL5 medium (Fig. 2C). We observed that ppk1 mutant cells have a growth defect, with a mean generation time of 15 h, compared with 11 h for wild type (WT), demonstrating the importance of Ppk1 for *D. discoideum* physiology.

We next analyzed polyP content by resolving 90 μg of RNA/polyP extract from WT and ppk1 cells by PAGE, revealing the complete loss of the polyP negative stain in ppk1 (Fig. 2D). This analysis demonstrates that Ppk1 is in fact the only enzyme responsible for polyP synthesis in *D. discoideum* cells. Previous analysis of the level of polyP in ppk1 cells reported that polyP was merely reduced to 20–50% of the WT level (27). There are two explanations for this discrepancy: The first is the use of indirect assays to determine polyP levels. These assays developed using synthetic polyP fail to account for the effect of copurifying interfering molecules. PolyP extraction not only purifies polyP and RNA but also inositol phosphates (Fig. 2D), nucleotides (28), and likely free phosphate. The abundance of these phosphate-containing molecules influences these polyP quantification methods (8). The second explanation is that the previously generated ppk1 line deleted only 114 nt of 3,153 nt, just <3% of the Ppk1 ORF (27) and that this mutant retains active Ppk1 enzyme. This hypothesis is supported by the identification of PPK activity in this mutant (27).

Because polyP has been associated with primary metabolism (29) and that any change to Pi buffering seems likely to affect basic metabolism, we decided to investigate whether the level of ATP was affected. In fact, ppk1 cells displayed a 2.5-fold reduction in ATP, offering a simple explanation for the overall fitness defect of these cells (Fig. 2E). We next analyzed the level of polyP after inhibiting both the cytochrome mediated respiration (with KCN) and the alternative oxidase (AOX) pathway with benzohydroxamate (BHAM) (30). Sublethal doses of these drugs induced a ~40% decrease in the level of ATP in WT amoebae (Fig. 2G), but did not kill the cells during the 2-h time course. During this time course, we also observed an unexpected increase in polyP level (Fig. 2F). Conversely, ppk1 cells did not display any accumulation of polyP nor change in ATP levels (Fig. 2 H and I). Meanwhile, the levels of inositol pyrophosphate were unaffected by these treatments (Fig. 2 F and H).

**polyP Levels Increase During D. discoideum Development.** *D. discoideum* was originally selected as an experimental model to study the transition to multicellularity. Single cells undergo a defined

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*Fig. 1. D. discoideum polyP detected by PAGE analysis. Synthetic polyP standards with an average chain length of 25 (T25) and 65 (T65) were loaded, either untreated or after incubation with the exo- and endopolyphosphatase Ppx1 and Ddp1. Equal amounts of vegetative AX2 D. discoideum phenol chloroform extract, as normalized RNA (70 μg) were treated with DNase, RNase, exo-, and endopolyphosphatase (Ppx1 and Ddp1) or treated in acid at high temperature (acid boiled). Samples were analyzed by 20% PAGE and stained with DAPI. Phenol extract from wild-isolate NC4 cells (Right lane) revealed the same type of polyP. The negative stained smears depend on the polyP property to induce DAPI photobleaching (23). The signal disappears after Ppx1 and Ddp1 incubation or acid treatment, whereas the white fluorescence reveals RNA species degraded by RNase treatment. The results shown are of a representative experiment that was repeated more than three times.*

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developmental process through a multicellular “slug,” culminating in a spore-containing fruiting body. We decided to investigate polyP metabolism during the substantial physiological changes associated with *D. discoideum* development. Because the starvation response induces *D. discoideum* development, we shifted a vegetative growing culture from rich HL5 medium to nonnutrient (phosphate buffer) agar and collected cells at different time points corresponding to the diverse developmental stages. PAGE analysis of phenol extract from WT amoebae revealed an impressive accumulation of polyP during development (Fig. 3A). Densitometry scanning of Toluidine-stained gels (Fig. 3B), which although less sensitive, offers a better dynamic range (31), showed the increase in polyP between vegetative stage and fruiting body was more than 100-fold. The average size of polymers did not substantially change during this accumulation (Fig. 3A and B). Analysis of *ppk1* amoebae confirmed that Ppk1 is the enzyme responsible for this accumulation of polyP (Fig. 3A). Analysis of the wild-isolate NC4 strain confirmed the striking accumulation of polyP during the developmental progression is coherent with the increased expression of the Ppk1 gene during the late stages of development as reported by the DictyExpress database (https://dictyexpress.research.bcm.edu/landing/).

**Ppk1 Is Required for Spore Germination.** We did not observe any developmental phase delay between WT and *ppk1* cells. The number of fruiting bodies formed was unaffected in the *ppk1* background. The fruiting bodies of the mutant were correctly formed with identifiable basal disks, stalks, and spore heads.
However, the overall size of the fruiting bodies was considerably reduced (Fig. 4A). Because fruiting bodies consist of two cell types, we decided to investigate whether polyP accumulates in spores or stalk cells. We isolated pure preparations of spore cells and compared polyP levels extracted from spores to those extracted from whole fruiting bodies. We normalized between these samples by spore number and extracted from $5 \times 10^6$ spores in each case. This analysis revealed that a large proportion of polyP accumulates in the spore (Fig. 4B).

The storage of polyP in the spores suggested an important role for this polymer during spore germination, potentially acting as a store of phosphate, minerals, or energy. In fact, analysis of spore germination in rich HL5 medium revealed a germination delay in the mutant strain (Fig. 4C). The polymeric nature of polyP also offers clear osmotic benefit due to its ability to complex with counterions. Thus, polyP synthesis/degradation can also buffer osmotic pressure by capturing/realizing Pi and cations. We investigated the effect of osmotic stress on germination and found that the defect observed between WT and ppk1 cells was amplified in media supplemented with 0.2 M sorbitol (Fig. 4D).

**Altered ATP and Accumulation of Inositol Pyrophosphates in ppk1 Mutant Spores.** In yeast, the level of polyP is metabolically connected to the level of inositol pyrophosphates (17). These molecules (InsP$_7$ and InsP$_8$) are notable for their ability to control cellular ATP synthesis (32, 33). Because polyP influences the level of ATP in vegetatively growing cells (Fig. 2E), we wondered if the massive absence of polyP (and therefore phosphoanhydride bonds) during development of ppk1 cells (Fig. 3A) might affect inositol pyrophosphate synthesis or ATP production.

The analysis of inositol phosphates extracted from WT and ppk1 cells revealed that the ratio of InsP$_7$ and InsP$_8$ to their precursor InsP$_6$ is significantly altered (Fig. 5A). The quantification of several experiments revealed that ppk1 fruiting bodies accumulated more than twice the level of inositol pyrophosphates compared with WT (Fig. 5B). Although we were technically unable to quantify ATP in the later stages of D. discoideum development, we observed that during the initial phases of development ATP level in WT cells remained largely unchanged. Meanwhile, the level of ATP in ppk1 cells was substantially increased (Fig. 5C) and reached levels slightly exceeding those of WT amoebae. These results indicated that when polyP cannot be synthesized, as in ppk1, cells accumulated inositol pyrophosphates InsP$_7$ and InsP$_8$. This perhaps represents a partial compensation, in which energy is stored in phosphoanhydride bonds of inositol pyrophosphates rather than polyP. It is clear that the additional energy...
stored in the phosphoanhydride bonds of InsP7 and InsP8 in ppk1 will not be equal to that accumulated in WT polyP. However, these results do confirm a metabolic interplay between these two families of phosphate-rich molecules and their ability to regulate or be regulated by cellular energy metabolism.

Discussion

The ability of polyP to covalently modify proteins (13) and to work as a chaperone (12) have recently boosted interest in this polymer. Certainly polyP is no longer the “forgotten polymer” (34) it was a few years ago. The study of polyP has long been inhibited by the lack of reliable techniques, a fact not lost on Kornberg et al. who commented on “the inadequacy of methods to establish the authenticity and size of polyP” (8). Here we have coupled high-quality PAGE analysis with the sensitivity and specificity of DAPI staining to reliably detect polyP in D. discoideum. Furthermore, we were unable to detect any polyP in cells lacking Ppk1. Earlier work described the involvement of Ppk1 in fruiting body development as well as an effect on the predation behavior of the amoebae (27). In this study, we generated a ppk1 mutant amoeba by deleting ~70% of the Ppk1 ORF, revealing many previously unrecognized phenotypes.

A modest developmental increase of polyP was previously noted (27); however, our analysis has revealed an unprecedented >100-fold increase in the level of polyP during D. discoideum development. The astonishing polyP increase and accumulation in the spore is reminiscent of InsP6 (phytic acid) accumulation in plant seeds (35). These phosphate-rich molecules share similar biophysical characteristics, including the ability to chelate divalent cations. Therefore, as InsP6 is important to supply phosphate and cations during seed germination (35), polyP might play similar roles during spore germination as demonstrated by the reduction in germination efficiency in ppk1 cells.

Because polyP can act as a Pi donor and buffer, it follows that polyP might regulate ATP metabolism. In yeast, a genome-wide screen revealed the interdependence of polyP with primary metabolism (29). In mammalian cells, alteration of mitochondrial metabolism affects polyP production (36). It has also been suggested that polyP is an activator or even a constituent of the

Fig. 4. ppk1 cells show impaired fruiting body formation and spore germination. (A) Representative images of fruiting bodies from WT and ppk1 cells. Images were taken at the same magnification. (B) Analysis by 20% PAGE of polyP extracted from whole fruiting bodies and pure spore preps, stained with DAPI. Time courses of spore germination in H5L media (C) and H5L media supplemented with 0.2 M sorbitol (D) over 24 h. WT black line; ppk1 dashed line. The date represent the average ± SD of three experiments run in duplicate (*P < 0.05).

Fig. 5. Developmental failure to accumulate polyP affects inositol pyrophosphate and ATP metabolism. (A) Analysis by 35% PAGE of perchloric acid extract from WT and ppk1 cells during development, stained with Toluidine blue. (B) Quantification of the ratio between inositol pyrophosphates (InsP7 and InsP8) and their precursor, InsP6 in fruiting bodies. Quantification by densitometry using ImageJ software, results represent the average ± SD of three independent experiments (***P < 0.0001). (C) Quantification of fold change in ATP between vegetative cells and aggregating cells starved for 9 h. The data represent the average ± SD of six experiments run in duplicate (*P < 0.05).
mitochondrial permeability transition pore (37). Thus, it has been proposed that polyP plays a key role in defining the mitochondrial permeability transition transition pore (37). This counterintuitive results invite further work with D. discoideum to define the precise role of polyP in mitochondrial function and the mitochondrial permeability transition pore.

The compensatory increase of inositol pyrophosphates and ATP driven development is supportive of a model in which there is a functional interplay between inositol pyrophosphates, ATP, and polyP. This nascent hypothesis requires further work to be fully validated. However, our characterization of D. discoideum polyP metabolism and the development of ppk1 cells, offer the unique platform to investigate the link between polyP and ATP production. It is noteworthy to remember that inositol pyrophosphates were discovered in D. discoideum (33); thus it is the ideal experimental model to investigate the metabolic connection between these molecules. The yeast S. cerevisiae and the ppk1 amoebae are the only two eukaryotic models in which a genetic approach has eliminated polyP. Whereas both experimental models offer distinctive advantages, the larger size of amoebae, absence of a cell wall, and a polyP level more similar to mammalian cells, make D. discoideum an excellent model to dissect polyP functions and the ideal model to develop more specific probes for localizing polyP in the cell.

Materials and Methods

Detailed methods are provided in SI Materials and Methods.

Inorganic polyphosphate was extracted from cell pellets at indicated time points. Cells were resuspended in one volume of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris HCl, pH 8, 0.5% SDS), one volume of acidic phenol (pH 4.0) was added, and samples were vortexed at 4 °C for 5 min. Samples were spun at 17,000 × g for 5 min at 4 °C and aqueous phase was recovered. Two volumes of chloroform were added and samples were again vortexed at 4 °C for 5 min before spinning at 5,000 rpm for 5 min. The aqueous phase was collected and precipitated by adding 2.5 volumes of ethanol and incubating at −20 °C for 1 h. After precipitation, pellets were resuspended in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% SDS. RNA concentration was measured by nanodrop and used to normalize samples before loading on PAGE. Inositol pyrophosphates were extracted as described before (31).

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