Inorganic polyphosphate in *Dictyostelium discoideum*: Influence on development, sporulation, and predation

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*Dictyostelium discoideum*, a social slime mold that forms fruiting bodies with spores, depends on inorganic polyphosphate (poly P) for its cycles of development and for nutritional predation on bacteria. The synthesis of poly P, a polymer of tens or hundreds of phosphate residues linked by high energy, ATP-like bonds, is catalyzed in most bacteria by poly P kinase (PPK1). The eukaryote *D. discoideum* possesses a homolog of PPK1. We report here that mutants of *D. discoideum* PPK1 (DdPPK1) have reduced levels of poly P and are deficient in development. Fruiting bodies are smaller and produce fewer spores, which appear to germinate like the wild type (WT). The DdPPK1 mutant formed smaller plaques on bacterial lawns compared with those of the WT. Predation by *D. discoideum*, assessed by uptake and digestion of *Klebsiella aerogenes*, showed that fewer bacteria were taken up by the DdPPK1 mutant compared with the WT and were killed less rapidly, indicating a role of poly P and/or DdPPK1 in phagocytosis. On *Pseudomonas aeruginosa* lawns, cleared plaques were observed with the bacterial PPK1 mutant but not with the WT *P. aeruginosa*. Thus, poly P is important in predation both for the predator and prey.

Likely an agent in evolution from prebiotic times, inorganic polyphosphate (poly P) in chains of tens to hundreds of phosphate units linked by high-energy phosphoanhydride bonds is now found in volcanic condensates and deep oceanic steam vents and has been conserved in all cells: bacteria, fungi, plants, and animals. Poly P can be formed from Pi simply by dehydration at elevated temperatures. In eukaryotes, it is found in virtually all subcellular organelles, even at levels of 20 percent of the cell dry weight (1). Poly P is required for bacterial responses to a variety of stresses and stringencies and for the virulence of some pathogens (1–6). It is also involved in the proliferation of mammalian cells by stimulating the kinase activity of mammalian target of Rapamycin (7). Among many poly P-metabolizing enzymes, polyphosphate kinase (PPK) reversibly catalyzes the polymerization of the terminal phosphate of ATP to poly P (8). PPK homologs have been found in 80 or more prokaryotic species, including 17 pathogens, but in only one eukaryote, the social slime mold *Dictyostelium discoideum* (9).

The genes used to parasitize protozoa and macrophages are widely conserved. Thus, *Legionella pneumophila*, with no known animal host, switches on the same genes on entry into either amoebae or macrophages (10). Furthermore, they exit these eukaryotic predators with similar properties of virulence and resistance (11–13). Protozoa feed on bacteria by a phagocytic mechanism, similar to those used by higher eukaryotes, that involves attachment of bacteria to cell-surface receptors, influenced by relative surface hydrophobicity (14). Engagement of the bacteria depends on actin polymerization, membrane exocytosis, and formation of phagolysosomes. The regulation of protozoal cell motility, membrane trafficking, and internalization events resembles that of neutrophils and macrophages (15–17). That coevolution of bacteria and protozoa may have led to animal pathogens (18–21) has promoted the use of protozoa as convenient models for virulence pathways.

*D. discoideum* has been used to study host–pathogen interactions, in particular for *Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa* (18, 22–25). With a small haploid genome, *D. discoideum* is a genetically tractable host and undergoes a developmental cycle. When nutrients are available, either in the form of bacteria or an axenic medium, the cells grow vegetatively as amoebae. When starved, amoebae use a CAMP signal relay to stream into aggregates of up to $10^7$ cells and finally form a fruiting body that contains stalk cells and spores that can germinate to complete the cycle (15). From amoebae to spores, poly P is found in every stage of development of *D. discoideum* (26), mostly in vacuoles called acidocalcisomes (27). Sims and Katz first observed that resistance of *D. discoideum* mutants to polyene antibiotics mapped in a genetic locus homologous to *E. coli* PPK1 and observed that the mutant is abnormal in development (M. Sims and E. Katz, personal communication). We have confirmed that the *D. discoideum* homolog is indeed a PPK1 (*D. discoideum* PPK1, DdPPK1), and we found that null mutants are defective in development, sporulation, and predation.

Materials and Methods

**Cells and Growth Conditions.** The *D. discoideum* cell line includes wild type (WT) (AX2) and mutant AX2M1 [AX2 Δdppk1::Bsr (Blasticidin resistance)] (see below). All strains were grown at 21°C in HL5 (28) medium. Cells were also grown in association with *Klebsiella aerogenes* on SM5 agar plates (29). *P. aeruginosa* WT PAO1 and mutant PAOM5 [PAO1 Δppk1::tet (Tc²)] (2) were grown in LB at 37°C. Antibiotics were Blasticidin, 5 μg/ml (13); G418, 10 μg/ml (30); and tetracycline, 15 μg/ml.

**Mutant Construction.** Two segments of *Ddppk1* (GenBank accession no. AF176830) were amplified from *AX2* genomic DNA by PCR. Primer 5U with a XhoI site (in bold) (CCCGTCTAGATGGCATATTGATTTTGA) and 5.5 μl with a HindIII site (in bold) (CCCCAGCTTTTTCCAAGAGGCCGATGTTT) were used for the 5’ segment. Primer 3U with a XbaI site (in bold) (GGCTCTAGATGGCAATTGTGGATACACTC) and 3.5 μl with an EcoRI site (in bold) (GGGAATTCGCTTTACCTTCTGTGGGCTT) were used for the 3’ segment. The knock-out plasmid pSP72-Ddppk1-Bsr was obtained by inserting the 5’ segment of *Ddppk1* into pSP72-BSr (31) between the XhoI and HindIII sites at the 3’ end of the *brr* (blasticidin resistance) gene, and the 3’ segment of *Ddppk1* into XhoI and EcoRI sites at the 5’ end of *brr*. pSP72-Ddppk1-Bsr was digested by *XhoI* and *EcoRI*, the 4.3-kb fragment containing *Ddppk1* segments on both ends of *brr* was recovered and transformed into *AX2* by electroporation. Transformed cells were selected by resistance to 5 μg/ml blasticidin. Individual clones were screened by PCR with primers 5U, 3L, and several other primer sets for the correct integration of *Ddppk1*:Bsr. One of the clones, AX2M1 (AX2 Δdppk1::Bsr), was used for further study.

Abbreviations: poly P, inorganic polyphosphate; PPK, polyphosphate kinase; DdPPK, *Dictyostelium discoideum* PPK.

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**Developmental Assay.** Multicellular development was examined on *K. aerogenes* lawns or on nitrocellulose (NC) filters. For development on *K. aerogenes* lawns, *D. discoideum* was grown to mid-log phase in HL5 medium; 10⁶ or 10³ cells were mixed with 0.2 ml of overnight culture of *K. aerogenes* and plated on SM5 plates. Pictures of plaques and fruiting body formation were taken at various times. After the fruiting bodies were fully thawing. After centrifugation at 13,000 × g at 4°C for 10 min, the supernatant (crude lysate) was used for the PPK1 reaction. The reaction mixture (25 µl) contained 50 mM HEPES (pH 7.2), 13 mM KH₂PO₄, 4 mM MgCl₂, 0.5 mM poly P (Sigma type 75, in phosphate residues), 1 mM ATP, 1 mM creatine phosphate, and 20 µg/ml creatine kinase.

**Biochemical Assays.** Poly P was extracted and determined by both radioactive and nonradioactive methods (3, 32). The PPK1 assay for *D. discoideum* was performed as described in ref. 8 with modified reaction conditions. Cells were lysed by freezing-thawing. After centrifugation at 13,000 × g at 4°C for 10 min, the supernatant (crude lysate) was used for the PPK1 reaction. The reaction mixture (25 µl) contained 50 mM HEPES (pH 7.2), 13 mM KH₂PO₄, 4 mM MgCl₂, 0.5 mM poly P (Sigma type 75, in phosphate residues), 1 mM ATP, 1 mM creatine phosphate, and 20 µg/ml creatine kinase.

**Table 1.** Poly P content of *D. discoideum* cells at different stages

<table>
<thead>
<tr>
<th>Vegetative growth stages</th>
<th>Poly P content, nmol/mg protein</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>4.9</td>
</tr>
<tr>
<td>Early stationary</td>
<td>11.3</td>
</tr>
<tr>
<td>Stationary</td>
<td>15</td>
</tr>
<tr>
<td>Spore</td>
<td>59</td>
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</table>

*Poly P content is calculated in phosphate residues. Numbers were the average of several experiments.*

**Results**

**Ddpk1, the Gene and Mutant.** *D. discoideum* contains a homolog of *Escherichia coli* ppk1, designated Ddpk1. Ddpk1 has 3,153 base pairs without an intron, the only bacterial ppk1 homolog yet found in eukaryotic cells. The deduced amino acid sequence shares 30% identity and 51% homology with *E. coli* PPK1. However, *E. coli* PPK1 contains only 688 amino acids compared with 1,050 amino acids in Ddpk1; about one-third of the N-terminal sequence has no homology with *E. coli* PPK1.

**Fig. 1.** Growth of *D. discoideum* in HL5 medium.

**Fig. 2.** Growth of *D. discoideum* on a *K. aerogenes* lawn. (A) *D. discoideum* cells (1 × 10⁶) were mixed with *K. aerogenes* and plated on SM5 plate. Plaques were formed by WT (Left) and mutant (Right) cells on lawns after 2 days. (B) *D. discoideum* cells (20–50) were mixed with *K. aerogenes* and plated on SM5 agar. Plaque sizes were measured over a 7-day period.
Moreover, the deduced protein has an asparagine (N818) in place of one of the essential histidines (H460) of E. coli PPK1.

Low PPK1 activity (200 units/mg protein) in extracts of the WT under standard reaction conditions was increased 15-fold by the addition of poly P and optimizing pH and salt concentrations. At the levels of 3,600 units/mg protein, the optimized activity exceeded that of the PPK1 value of 2,000 in an E. coli lysate. When grown in HL5 medium, the DdPPK1 activity peaked at mid-log phase and declined when the cells entered stationary phase.

A mutant of Ddpk1 that disrupts the single copy of Ddpk1 in the haploid D. discoideum genome was constructed (see Materials and Methods). The coding sequence from 1591 to 1705 was replaced by a bsr gene. The correct deletion-insertion mutation allele of Ddpk1 was confirmed by PCR with at least four sets of primers. PPK1 activity in the mutant was only 5% that of the WT in all six individual mutant clones tested; one of these clones, AX2M1, was used in these studies.

Poly P levels in vegetative cells were highest in the stationary phase but far lower than in spores (Table 1). The chain lengths that of the WT in all six individual mutant clones tested; one of four sets of primers. PPK1 activity in the mutant was only 5% of the WT level.

Growth Defect of Mutant on Bacterial Lawns. The mutant was severely deficient in growth on bacterial lawns measured by the size of plaque formed by single cells on K. aerogenes, although growth appeared the same for the WT and mutant in HL5 medium (Fig. 1). When mid-log phase cells were diluted into an overnight K. aerogenes culture and plated on SM5 agar plates, the average size of a plaque after 7 days was only about 1/10th the size for the mutant compared with the WT (Fig. 2); complementation of the mutant restored growth to the WT level (Fig. 2). Similar results were obtained on lawns of the P. aeruginosa PPK1 mutant PAOM5 (data not shown).

Defects of Mutant in Development. The DdPPK1 mutant was delayed in delaying body formation when examined on a K. aerogenes lawn (Fig. 3) because of slower growth. WT cells were fully developed into fruiting bodies in 3–4 days, but those of the mutant were only about one-fifth the size of the WT even after 7 days (Fig. 3). The spore number produced by the mutant was only ~20% that of WT (Table 2). Growth of the complemented mutant was similar to that of the WT, it formed a normal-sized fruiting body (Fig. 3), and produced many spores (Table 2).

With development on filters, the mutant was slightly delayed and formed smaller fruiting bodies as compared with the WT and could also be complemented as above (data not shown).

During germination, the poly P level in the WT dropped dramatically before amoebae emerged and remained constant thereafter (Fig. 4), whereas the mutant, which had not accumulated poly P, showed only a small decrease. Yet, the levels of poly P did not appear to affect the germination as measured in HL5 medium (Fig. 4).

Role of DdPPK1 in Phagocytosis and Digestion. The reduced ability of the mutant DdPPK1 to feed on bacterial lawns could be due to defects in phagocytosis, in endosome processing, or in motility. To appraise phagocytosis, a modified gentamicin-protection assay was used to measure the uptake and digestion rate by K. aerogenes. The WT and mutant were each incubated with K. aerogenes for 30 min; bacteria that was not taken up by the amoebae were killed by the addition of gentamicin. One hour after the addition of gentamicin, 1.4 × 10^8 K. aerogenes cells were detected inside the WT, but only 5% as many (8 × 10^7 cells) were inside the mutant (Fig. 5), indicating reduced phagocytosis by the Dictyostelium mutant. After 2 hours more, digestion of bacteria by the WT left only 1% of the phagocytosed bacteria viable, whereas 15% of the bacteria were still detected in the mutant D. discoideum. Thus, during the 2-hour interval, the WT digested ~10 times as many bacteria as did the mutant, sugges-

Table 2. D. discoideum Ddpk1 mutant shows reduced sporulation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sporulation,* %</th>
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<tbody>
<tr>
<td>WT</td>
<td>100</td>
</tr>
<tr>
<td>Mutant</td>
<td>20</td>
</tr>
<tr>
<td>Mutant + Ddpk1</td>
<td>135</td>
</tr>
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D. discoideum cells (1 × 10^6) were mixed with K. aerogenes and plated on SM5 agar plates. Spores were collected on the fourth day for AX2 (WT) and AX2M1 (act15/Ddpk1) (mutant + Ddpk1) and on the seventh day for AX2M1 (mutant). Spore numbers were counted in a hemocytometer.

*Sporulation refers to the number of spores produced by a strain as a percentage of the number of spores produced by the WT.
P level is crucial in the predator–prey relationships of the delayed in the mutant (Fig. 3), and sporulation is diminished cells and spores (Table 1). Development of fruiting bodies is homologue has been found in a eukaryote, the social slime mold as PPK1. In this article, we describe an instance in which a PPK1 a wide range of bacterial species is a poly P kinase, designated The enzyme responsible for the synthesis of poly P from ATP in between predator and prey.

Further Role of Poly P and/or PPK in Predator–Prey Relations. The role of poly P and/or PPK in the contest between predator and prey can be observed in the outcome of interactions between P. aeruginosa and D. discoideum. When P. aeruginosa WT (PAO1) and Δppk1 (PAOM5) were used in the plaque formation assay with the WT D. discoideum, the P. aeruginosa WT was taken up by the amoebal form of D. discoideum and killed them, leaving an intact bacterial lawn. However, on a lawn of Δppk1, the WT D. discoideum proved to be an effective predator as observed by the plaques formed (Fig. 6). The mutant D. discoideum also forms plaques on the Δppk1 lawn, but they are smaller than the WT. Thus, poly P and/or PPK1 are crucial in the balance between predator and prey.

Discussion
The enzyme responsible for the synthesis of poly P from ATP in a wide range of bacterial species is a poly P kinase, designated as PPK1. In this article, we describe an instance in which a PPK1 homologue has been found in a eukaryote, the social slime mold D. discoideum. Mutants lacking the enzyme (DdPPK1) are defective in development, sporulation, and predation. Poly P levels in the DdPPK1 mutant are reduced both in the vegetative cells and spores (Table 1). Development of fruiting bodies is delayed in the mutant (Fig. 3), and sporulation is diminished (Table 2); these developmental deficiencies can be overcome by complementation with the Ddppk1 gene.

A striking effect of the lack of PPK1 and the reduction in poly P level is crucial in the predator–prey relationships of the D. discoideum.

Fig. 5. Uptake and digestion of K. aerogenes by D. discoideum cells. WT and mutant cells (1 × 10⁶ per ml) were plated in tissue culture wells and infected with K. aerogenes at a multiplicity of −100:1. Cultures were incubated at 22°C for 30 min, at which time gentamicin (Gm) was added to kill extracellular K. aerogenes. D. discoideum were collected at indicated time points, lysed, and plated on nutrient agar plates to determine the colony-forming units (cfu)/ml D. discoideum cell lysates.

Fig. 6. Plaque formation of D. discoideum cells on P. aeruginosa lawns. WT cells were mixed with P. aeruginosa WT (Left) and mutant (Right) and plated on SM5 agar and incubated at 22°C for 5 days.