The inositol pyrophosphate metabolism of *Dictyostelium discoideum* does not regulate inorganic polyphosphate (polyP) synthesis

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ABSTRACT

Initial studies on the inositol phosphates metabolism were enabled by the social amoeba *Dictyostelium discoideum*. The abundant amount of inositol hexakisphosphate (IP$_6$) also known as Phytic acid) present in the amoeba allowed the discovery of the more polar inositol pyrophosphates, IP$_7$ and IP$_8$, possessing one or two high energy phosphoanhydride bonds, respectively. Considering the contemporary growing interest in inositol pyrophosphates, it is surprising that in recent years *D. discoideum*, has contributed little to our understanding of their metabolism and function. This work fulfills this lacuna, by analysing the ip6k, ppip5k and ip6k-ppip5K amoeba null strains using PAGE, $^{13}$C-NMR and CE-MS analysis. Our study reveals an inositol pyrophosphate metabolism more complex than previously thought. The amoeba Ip6k synthesizes the 4/6-IP$_7$ in contrast to the 5-IP$_7$ isomer synthesized by the mammalian homologue. The amoeba Ppip5k synthesizes the same 1/3-IP$_7$ as the mammalian enzyme. In *D. discoideum*, the ip6k strain possesses residual amounts of IP$_7$. The residual IP$_7$ is also present in the ip6k-ppip5K strain, while the ppip5k single mutant shows a decrease in both IP$_7$ and IP$_8$ levels. This phenotype is in contrast to the increase in IP$_7$ observable in the yeast vip1Δ strain. The presence of IP$_7$ in ppip5k and the presence of IP$_7$ in ip6k-ppip5K indicate the existence of an additional inositol pyrophosphate synthesizing enzyme. Additionally, we investigated the existence of a metabolic relationship between inositol pyrophosphate synthesis and inorganic polyphosphate (polyP) metabolism as observed in yeast. These studies reveal that contrary to the yeast, Ip6k and Ppip5k do not control polyP cellular level in amoeba.

1. Introduction

The social amoeba *Dictyostelium discoideum* was one of the primary experimental models to study inositol phosphate metabolism and signalling in the 1980s (Europe-Finner et al., 1991). The lipid independent route to IP$_6$ synthesis was identified (Stephens and
Irvine, 1990) in *D. discoideum*, and inositol species more polar than the fully phosphorylated ring of IP$_6$, the inositol pyrophosphate (see below) were also discovered in this organism (Stephens et al., 1993). By the middle of the 90s, it was discovered that phospholipase C in *D. discoideum* is not required to produce IP$_3$ nor to control calcium signalling (Van Dijken et al., 1995, 1997). Perhaps for these reasons, the interest of inositol scientists in this organism faded over the years. However, the interest in *D. discoideum* did not completely disappear. At the start of the new millennium, the social amoeba was used to study the effect on the inositol phosphate metabolism of lithium and other mood stabilizing drugs (King et al., 2010; Williams et al., 1999, 2002). More recently, *D. discoideum* was used to characterise the roles of inositol polyphosphate in programmed cell death (Al-Anbaky et al., 2018) and to characterise the pytocannabinoid-dependent mTORC1 regulation by the inositol polyphosphate multikinase (Damstra-Oddy et al., 2021). However, the precise description of the inositol phosphate metabolic pathway and the characterization of the different inositol kinase mutants is still missing in the amoeba. To our knowledge, only the IP$_6$K (Ip6K) null strain (ip6k) has previously been generated. The previously characterized *ip6k* amoeba (Luo et al., 2003) possess a biochemical phenotype, the absence of inositol pyrophosphate, similar to the mutant of the homologous yeast *Saccharomyces cerevisiae* gene Kcs1 (kcs1Δ) (Saiardi et al., 2000).

The IP$_6$K, as the name indicates, phosphorylates IP$_6$ to generate the inositol pyrophosphate IP$_7$ (Saiardi et al., 1999). There is at least one other class of kinases able to synthesize inositol pyrophosphate known as PPIP5K (*S. cerevisiae* Vip1; *Schizosaccharomyces pombe* Asp1) that, as the name suggests, primarily phosphorylates IP$_7$, also abbreviated to PP-IP$_5$, IP$_8$ or (PP)$_2$-IP$_4$ (Choi et al., 2007; Fridy et al., 2007; Mulugu et al., 2007).

The yeast and mammalian IP$_6$K and PPIP5K have been extensively characterized. The mammalian IP$_6$K is able to phosphorylate position five of the inositol ring generating the isomer 5PP-IP$_6$ (hereafter 5-IP$_6$) (Draskovic et al., 2008), while mammalian PPIP5K5 phosphate position one physiologically generating 1,5(PP)$_2$-IP$_4$ (hereafter 1,5-IP$_4$) (Lin et al., 2009; Wang et al., 2012).

*D. discoideum* possesses one *ip6k* gene and a Ppip5k homologous gene (see below). The similar enzymology between human, yeast and amoeba suggests a similar inositol pyrophosphate metabolism, however, this appears not to be the case since $^{1}$H, $^{31}$P-NMR spectroscopy and enzymology studies (Laussmann et al., 1996, 1997) suggest that the amoeba possesses a different form of IP$_6$, the isomer 5,6(PP)$_2$-IP$_4$ (hereafter 5,6-IP$_4$).

Inositol pyrophosphates are attracting a growing interest due to their link to metabolic disorders including obesity and diabetes (Mukherjee et al., 2020), human diseases, such as cancer and Alzheimer’s (Crocco et al., 2016), combined with improved tools to facilitate their analysis in vivo (Harmel et al., 2019; Ito et al., 2018; Qiu et al., 2020; Wilson et al., 2015). The picture that is emerging is that inositol pyrophosphates regulate basic energy metabolism (Szijgyarto et al., 2011) through their ability to control phosphate homeostasis (Azevedo and Saiardi, 2017) and polyP synthesis is an evolutionarily conserved feature.

Nevertheless, the absence of polyP in *S. cerevisiae kcs1Δ* has been highly influential. In fact, *D. discoideum* *ip6k* knockout has been utilized as a proxy, using unrefined supporting data, which demonstrates that the amoeba has low polyP level (Suess and Gomer, 2016). Amoeba synthesizes polyP through the polyphosphate kinase (Ppk1), a gene acquired from bacteria by horizontal gene transfer (Livermore et al., 2016). We discovered that polyP hugely accumulates during the developmental program (Livermore et al., 2016). The polyP-induced synthesis following the starvation/aggregation signal leads to its secretion (Al-Anbaky et al., 2018) and to characterise the phytocannabinoid-dependent mTORC1 regulation by the inositol polyphosphate multikinase (Damstra-Oddy et al., 2021).

Like inositol pyrophosphates, polyP is also attracting a growing interest. PolyP, has been described as an important primordial chaperone able to regulate the aggregation of proteins which form in neurodegenerative disorders (Cremers et al., 2016). The polyP-induced synthesis following the starvation/aggregation signal leads to its secretion (Al-Anbaky et al., 2018) and to characterise the phytocannabinoid-dependent mTORC1 regulation by the inositol polyphosphate multikinase (Damstra-Oddy et al., 2021).

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Like inositol pyrophosphates, polyP is also attracting a growing interest. PolyP, has been described as an important primordial chaperone able to regulate the aggregation of proteins which form in neurodegenerative disorders (Cremers et al., 2016). However, it is also important to mitochondrial physiology (Solesio et al., 2021) driving a novel protein post translational modification, lysine-polypolyphosphorylation (Azevedo et al., 2015); and controlling several aspects of the blood coagulation cascade (Morrissey et al., 2012). Since the many important roles attributed to polyP, it is of fundamental importance to understand if the link between inositol polyphosphates (either IP$_7$ or IP$_9$) and polyP synthesis is an evolutionarily conserved feature.

*D. discoideum* offers the unique opportunity to address these issues since it is an excellent experimental model for both inositol pyrophosphate and polyP studies (Desfougeres and Saiardi, 2020). Here we characterise the amoeba inositol pyrophosphate metabolic pathway by creating the *ip6k*, the *ppip5k* and the double *ip6k-ppip5k* strain in the AX2 genetic background to verify if these enzymes are regulating polyP synthesis.

2. Materials and methods

2.1. Identification *D. discoideum* inositol phosphate kinase genes

To identify the amoeba inositol phosphate genes, we performed Protein Basic Local Alignment Search Tool (BLAST) searches using all inositol phosphate kinases found in *S. cerevisiae* and *H. sapiens* against the *D. discoideum* complete genome as previous described
2.2. Genetic manipulations

Yeast transformations were performed using the lithium/acetate method (Gietz and Woods, 2002). Yeast knockouts were generated using well-described procedures (Janke et al., 2004). The correct removal of the genes were first verify by PCR and then phenotypically characterising the inositol phosphate profile by $^3$H-inositol-labeling Sax-HPLC analysis (Azevedo and Saiardi, 2006).

2.3. Cloning *D. discoideum* Ppip5K in yeast expression vector

Codon-optimisation of *D. discoideum* Ppip5K sequences for yeast expression was designed through an interface from SciTools® (Integrated DNA Technology). Restriction sites were added at the 5′ *Sal*I and 3′ *Not*I to cloned Ppip5K in a pADH-GST plasmid (Azevedo et al., 2015).

2.4. Growth of yeast and amoeba

Yeast were grown in rich (YPD: 1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete medium (SC, Formedium) in the absence or presence of uracil to select for auxotrophy. For $^3$H-inositol labelling, the cells were grown in inositol-free media (SC-inositol, Formedium). The list of the yeast strains used in this study is given in Table 1. *D. discoideum* lines were isogenic to the axenic strain AX2. Amoeba were cultivated at 22 °C in HL5 media, either in Petri dishes or in flasks at 120 rpm. Cells were diluted every 1–2 days to avoid confluence of dishes or when cell densities exceeded $5 \times 10^6$ cells/ml.

2.5. Quantification of the PHO pathway activation by fluorescence-activated cell sorting

Logarithmic growing yeast grown in Sc-Ura media carrying pADH-Ppip5k or empty vector were washed and shifted in media with or without phosphate 10 μM for 3 h. Before FACS measurement, 50 μl of the yeast culture was diluted into 950 μl of TBS and immediately analysed using an LSRII flow cytometer (BD Biosciences).

2.6. $^{13}$C-NMR analysis

*D. discoideum* AX2 and ppip5k were grown for 5–7 days in SIH media (Formedium) supplemented with $^{13}$C$_6$-inositol (400 μM) synthesized as described (Harmel et al., 2019). Amoeba extracts were separated by PAGE to purify IP$_7$ and IP$_8$. In brief, whole-cell extracts from 350 ml labelled cultures were extracted with perchloric acid and run on single-lane 33% PAGE gels. Bands corresponding to each inositol pyrophosphate species were cut and elute over 24 h by rotation in alternating solutions of water and 1:1 water/methanol (Loss et al., 2011). The combined solutions were acidified with 0.1M perchloric acid and inositol pyrophosphate recovered by TiO$_2$ purification as described (Wilson et al., 2015). The $^{13}$C,$^1$H-NMR analysis was performed as previously described (Harmel et al., 2019) using a Bruker AVANCE III spectrometer.

2.7. Extraction and analysis of inositol polyphosphates form yeast

Logarithmic growing yeast were inoculated at OD$_{600}$ = 0.01 in 5 ml of SC-Ura-inositol supplemented with 5 μCi/ml of $^3$H-inositol. The yeast were grow for 16–20 h at 30 °C with shaking. Radiolabelled inositol phosphates were extracted and analysed by Sax-HPLC as described (Azevedo and Saiardi, 2006).

2.8. Extraction and PAGE analysis of inositol polyphosphates and polyP from amoeba

*D. discoideum* cells centrifuged (500 g, 4 min, 4 °C) and washed once with KK2 (20 mM potassium phosphate pH 6.8). Pellets were resuspended in 40–100 μl perchloric acid (1M), incubated in ice and vortexed for 10 s every 2 min for a total period of 10 min. The extracts were centrifuged at (18000 g, 5 min, 4 °C) and the supernatants were neutralised with 1M potassium carbonate supplemented

Table 1

| Name       | Relevant genotype                          | Reference               |
|------------|--------------------------------------------|-------------------------|
| DD1810     | MATa leu2 ura3-52 trp1 phb1-1122 pep4-3 pre1-451 | Onnebo and Saiardi, (2009) |
| kcs1Δ      | DD1810 KCS1::LEU2                          | Onnebo and Saiardi, (2009) |
| vip1Δ      | DD1810 VIP1::KANMX4                        | Onnebo and Saiardi, (2009) |
| kcs1Δvip1Δ | DD1810 KCS1::LEU2 VIP1::KANMX4             | Onnebo and Saiardi, (2009) |
| kcs1Δdp1Δ  | DD1810 DIP1::LEU2 KCS1::KANMX4             | This study               |
| EV1109     | MATa leu2::PHO84::GFP::LEU2 ADE2           | Thomas and O’Shea, (2005) |
| EV1109 pho81Δ | MATa leu2::PHO84::GFP::LEU2 ADE2 PHO81::NatNT2 | Desfougères et al., (2016) |
| EV1109 vip1Δ | MATa leu2::PHO84::GFP::LEU2 ADE2 VIP1::NatNT2 | This study               |

References:
- Laha et al., 2021
- Gietz and Woods, 2002
- Janke et al., 2004
- Azevedo and Saiardi, 2006
- Azevedo et al., 2015
- Azevedo and Saiardi, 2006
- Onnebo and Saiardi, 2009
- Onnebo and Saiardi, 2009
- Wilson et al., 2015
- Harmel et al., 2019
- Desfougères et al., 2009
- Thomas and O’Shea, 2005
- Desfougères et al., 2016
- Loss et al., 2011
with 3 mM EDTA at 4 °C for 2 h and subsequently centrifuged. For polyP analysis cell were extracted using acidic phenol procedure (Livermore et al., 2016). PAGE analysis over 33% acrylamide gel was performed as previously described (Losito et al., 2009). Briefly, gels were pre-run for 30 min before loading samples and running overnight at 700 V and 5 milliamps at 4 °C until the Orange G dye had run through 2/3 of the gel. Gels were stained by toluidine blue solution (20% methanol, 2% glycerol, 0.05% Toluidine Blue) at room temperature for 30 min with gentle agitation. Toluidine gels were destained twice in 20% methanol and scanned with an Epson Perfection 4990 Photo Scanner. Image quantification was carried using Image-J software package.

2.9. Generation of D. discoideum null strains

The ip6k and ppip5k strain were constructed using the TMO1 deletion vector (Muramoto et al., 2012). Regions of DNA flanking the gene of interest were amplified by PCR from AX2 genomic DNA using the oligo listed in Table 2. Knockout plasmids TMO1-Ip6K-Bsr, TMO1-PPip5K-Bsr, were generated by inserting these sequences into the plasmid TMO1, using NotI and EcoRI sites for the 5' arm and the HindIII and KpnI sites for the 3' arm. The resultant plasmids were then digested using BsuIII and used to transform AX2 cells by electroporation using a BioRad Inc. genepulser and exposed to a single pulse of 0.65 kV at 25 μF. Amoebas were subjected to blastocidin selection before screening the transformants by PCR, southern and northern blot and biochemically by PAGE analysis. To generate the ip6k-ppip5k strain the Blastocidin Resistance gene was excised from the pppip5k strain using the pDex-Cre-NLS plasmid (dictybase stock centre (Faix et al., 2004), before knocking out the Ip6k gene using the strategy described above. Southern blotting and Northern blotting were performed using a standard procedure.

2.10. D. discoideum development

Amoebas were starved by transferring cells from rapidly dividing vegetative cultures onto KK2 2% agar plates. 1 × 10^7 cells were plated on a 35 mm plate. Cells were allowed to develop at room temperature. Cells were collected after 1 h, ~8 h when cells were beginning to aggregate, ~16 h, when cells had coalesced to form slugs and after ~24 h when fruiting bodies were fully formed. For development on filters, three Whatman® Grade 3 filter papers were layered and covered by a Whatman® Grade 50 quantitative filter paper (hardened low-ash). The filters were soaked in the specified buffer. Following the removal of excess liquid, cells were resuspended in 500 μl of buffer (KK2, TrisHCl 20 mM pH7.0 and HEPES 20 mM pH7.0) and allowed to flow into the filter by delivery with a pipette in an outward spiral movement. Cells were harvested with a plastic scraper. Fruiting body images were then taken on an Olympus camera mounted on a dissecting microscope.

2.11. D. discoideum CE-MS analysis

D. discoideum extract for CE-MS analysis were prepared from vegetative growing strains in HL5 medium. Cultures (20–30 ml of 1–3 X 10^6 cell/ml) were spun at 500 g for 5 min; the cell pellet was washed in 1 ml KK2 and the inositol phosphate were extracted with 500 μl of perchloric acid 1M in the presence of 5 mM EDTA. The inositol phosphates in the perchloric acid extract were purified using TiO₂ (Wilson et al., 2015) before subjecting them to CE-MS-Q-TOF analysis. The analysis was performed as previously described with internal standards (Qiu et al., 2020) using an Agilent 7100 capillary electrophoresis system coupled to a Q-TOF (6520, Agilent) mass spectrometer. Data were collected with Agilent OpenLAB CDS Chemstation 2.3.53 and Agilent MassHunter Workstation Acquisition B.04.00.

3. Results and discussion

3.1. Identification of D. discoideum inositol phosphate kinases

The screening of D. discoideum genome revealed the presence of seven inositol phosphate kinases (Table 3). Out of these, only the Ip6k (gene name i6kA) has been characterized through the generation of the ip6k (i6kA) strain (Luo et al., 2003). Two other genes, LpkA and LpmK, were used in overexpression studies but not biochemically characterized (Damstra-Oddy et al., 2021; King et al., 2010). As opposed to the four kinases found in yeast (Laha et al., 2021; Saiardi et al., 2018; Tsui and York, 2010), the seven D. discoideum kinases pointed towards a higher complexity of inositol phosphate synthesis in the amoeba, more similar to mammalian cells. Indeed, like the

Table 2

| Oligo use to generate the Ip6K and Ppip5k deletion constructs. | Gene | Primer | Primer sequence | Product length | Restriction site |
| --- | --- | --- | --- | --- | --- |
| Ip6k | 5' arm Forward | GCACGCGCCCGCTCAATCCACCCACACCTCAC | 1050 | NotI |
| 5' arm Reverse | GCAGAATTCTGGTGTGTTGGCCTCTTGG | 835 | HindIII |
| 3' arm Forward | GCGAGCTGGGATAGTAGAATCCATACCTTC | 5' arm Forward | GCACGCGCCCGCTCAATCCACCCACACCTCAC | 1069 | NotI |
| 3' arm Reverse | GCAGGATCCCCTCTTGAGAGGACGACCTATGC | 832 | HindIII |
| Ppip5k | 5' arm Forward | GCACGCGCCCGCTCAATCCACCCACACCTCAC | 1069 | NotI |
| 5' arm Reverse | GCAGAATTCTGGTGTGTTGGCCTCTTGG | 835 | HindIII |
| 3' arm Forward | GCGAGCTGGGATAGTAGAATCCATACCTTC | 5' arm Forward | GCACGCGCCCGCTCAATCCACCCACACCTCAC | 1069 | NotI |
| 3' arm Reverse | GCAGGATCCCCTCTTGAGAGGACGACCTATGC | 832 | HindIII |
human genome, amoeba, possess Itpk1, the enzyme which drives the cytosolic route of IP₆ synthesis (Desfougeres et al., 2019). The amoeba also possesses one IP5-2Kinase (yeast Ipk1 and mammalian IPPK) and one PPIP5K gene. The inositol kinase enzymology of amoeba is similar to the mammalian counterpart, therefore it is peculiar, as stated in the introduction, that amoeba and human inositol pyrophosphate species differ in their isomeric nature. We, decided to reinvestigate this issue by performing the new structural studies using the newly developed ¹H,¹³C-NMR approach.

3.2. ¹³C-NMR characterization of D. discoideum IP₇ and IP₈

Previous NMR studies of IP₇ and IP₈ purified from amoeba were performed using two-dimensional ¹H,³¹P-NMR (Laussmann et al., 1996). This approach has limited sensitivity. Conversely, the newly developed ¹H,¹³C-NMR offers higher sensitivity since the chemical shift dispersion of ¹³C is superior to ³¹P, and the magnetization transfer via ¹H(¹H,¹³C) one-bond couplings is more efficient (Harmel et al., 2019). We fed wild type AX2 amoeba with ¹³C₆-inositol and after extracting and purifying the inositol pyrophosphates using TiO₂, we analysed them using ¹H,¹³C-NMR spectroscopy. The 2-dimensional inverse H,C correlation spectra (Fig. 1) confirm previous studies. The IP₇ spectra reveal the carbon 4/6 split signal, while the IP₈ spectra additionally reveal the shift towards the left of the signal of carbon 5. These are the typical signatures of pyrophosphate moieties at these two carbons (Harmel et al., 2019). Therefore, the inositol pyrophosphate isomers present in the social amoeba are indeed the 4/6-IP₇ and the inositol pyrophosphate isomers present in the social amoeba are indeed the 4/6-IP₈. These are the typical signatures of pyrophosphate moieties at these two carbons (Harmel et al., 2019). We fed wild type AX2 amoeba with ¹³C₆-inositol and after extracting and purifying the inositol pyrophosphates using TiO₂, we analysed them using ¹H,¹³C-NMR spectroscopy. The 2-dimensional inverse H,C correlation spectra (Fig. 1) confirm previous studies. The IP₇ spectra reveal the carbon 4/6 split signal, while the IP₈ spectra additionally reveal the shift towards the left of the signal of carbon 5. These are the typical signatures of pyrophosphate moieties at these two carbons (Harmel et al., 2019). Therefore, the inositol pyrophosphate isomers present in the social amoeba are indeed the 4/6-IP₇ and the 4/6,5-IP₈ forms. Of note, neither the myo-inositol 4/6 carbon positions nor the 1/3 carbon positions can be distinguished by NMR as they are enantiotopic. The different inositol pyrophosphate isomers present in amoeba and mammals, despite similar enzymology, suggests that the IP6K or the PPIP5K enzyme could pyro-phosphorylate different inositol ring positions depending on the species analysed.

3.3. D. discoideum Ppip5K rescues yeastvip1Δ phenotypes

To gain further insight into D. discoideum inositol pyrophosphate metabolism, we focused our attention on Ppip5k. The Ppip5k (DDB_G0284617) homologue in D. discoideum encodes a 56 kDa protein, compared to the 130 kDa yeast protein and ~150 kDa in mammalian cells (Fig. 2A). In both yeast and mammals, the PPIP5K encodes a protein containing both a kinase domain and a phosphatase domain (Dollins et al., 2020; Pascual-Ortiz et al., 2018). Interestingly, the D. discoideum gene encodes a much smaller enzyme, which completely lacks the phosphatase domain. The absence of this phosphatase domain in the amoeba might abolish the futile cycle proposed for this type of kinase (Randall et al., 2020).

The D. discoideum proteome has evolved to encode peptides with long poly-glutamine or poly-asparagine tracts (Santarriaga et al., 2015). The Ppip5k coding sequence possesses two long poly-asparagine stretches that have prevented us obtaining recombinant Ppip5k from bacterial expression systems. Similarly, D. discoideum Ip6k (DDB_G0278739) contains six poly-asparagine and one poly-glutamine repeats, which have also prevented us obtaining recombinant protein. Therefore, to test the amoeba Ppip5k activity, we cloned the gene into a yeast expression vector and transformed it into an array of strains.

Table 3: Inositol phosphate kinases in Dictyostelium discoideum.

| Gene ID     | Dicty base name | Gene name | Inositol Kinase Family | Chr. | Yeast homol. | Mammalian homol. | Ref.               |
|-------------|-----------------|-----------|------------------------|------|--------------|------------------|--------------------|
| DDB_G0281737 | -               | Ipmk      | IPK superfamily        | 3    | Arg32        | IPMK             | Damstra-Oddy et al. (2021) |
| DDB_G0278739 | I6kA            | Ip6k      | IPK superfamily        | 3    | Kcs1         | IP6K 1-3         | Luo et al., (2003)       |
| DDB_G0271760 | ipkA1           | Ipka      | IPK superfamily        | 2    | –            | –                | King et al., (2010)      |
| DDB_G0283863 | -               | Ipkb      | IPK superfamily        | 4    | –            | –                | –                  |
| DDB_G0269746 | Ipkl1           | Ipkl1     | ITPK1 family           | 1    | –            | ITPK1            | –                  |
| DDB_G0288351 | -               | Ipkl1     | IPPK family            | 5    | Ipkl1        | IPPK             | –                  |
| DDB_G0284617 | -               | Ppip5k    | PPIP5K family          | 4    | Vip1         | PPIP5K 1-2       | –                  |

To verify if the biochemical phenotype generates a product functionally equivalent to the Vip1 generated IP₇, we investigated if amoeba Ppip5k rescues the vip1Δ PHO response defect (Choi et al., 2017). In low phosphate conditions, a set of genes, named the PHO genes, are up-regulated. The expression of these genes is repressed in phosphate-rich conditions. One such gene is PHO84 that encodes a high affinity phosphate transporter. The induction of the PHO genes expression can be monitored by recording the expression of a reporter-protein cloned behind the PHO gene promoter. We took advantage of the strain (EY1109) developed by Thomas and O’Shea that expresses GFP under the control of the PHO84 promoter (Thomas and O’Shea, 2005). In this background, the deletion of Vip1 leads to a repression of the PHO genes expression, as previously demonstrated (Choi et al., 2017). This is similar to what is observed when PHO81 is deleted (Desfougeres et al., 2016). Expression of the amoeba Ppip5k in the vip1Δ strain fully rescues the expression of the reporter (Fig. 2C) indicating that, in vivo, the product of the enzymatic reaction catalysed by Ppip5k is functionally equivalent to...
the Vip1 product. The rescue of both biochemical and physiological vip1Δ phenotypes demonstrates that *D. discoideum* Ppip5k is a genuine PPIP5K enzyme able to phosphorylate 5-IP to, likely, 1,5-IP.

### 3.4. Generation of the *D. discoideum* ppp5k strain

Since NMR studies indicate that *D. discoideum* do not possess 1,5-IP (Laussmann et al., 1996) Fig. 1), we decided to knockout Ppip5k to characterise the effect of the absence of this kinase on the amoeba inositol pyrophosphate metabolism. A homologous recombination approach was used to generate amoeba knockout. This approach involved cloning two regions flanking the target gene
and inserting them on either side of a Blastocidin resistance marker gene. The strategy for deletion of the Ppip5k involved cloning 1.1 kb of genomic sequence overlapping slightly with the 5’ region of the gene and 0.8 kb 3’ of the gene (Fig. 3A). The generated ppp5k strains were confirmed by Southern blot (Fig. 3B); while Northern blot analysis (Fig. 3C) confirms the loss of the Ppip5K transcript in the ppp5k strain.

The analysis by PAGE of the ppp5k inositol pyrophosphates profile reveals a 49.6 ± 7.8% (n = 4) reduction in IP8 levels while a 14.3 ± 6.7% (n = 4) reduction in the level of IP7 is recorded (Fig. 3D). This biochemical defect is remarkably different from the one reported for yeast vip1Δ in which a substantial increase in IP7 is observed (Onmebo and Saiardi, 2009). The decrease of both IP7 and IP8 observed in ppp5k amoeba grown in rich HL5 medium, prompted us to verify if the reported increase on IP8 level during D. discoideum development (Laussmann et al., 2000; Pisani et al., 2014) is under Ppip5k control. The developmental analysis of inositol pyrophosphate profile in ppp5k amoeba reveals a consistent accumulation of IP8 during the late stage of development. In conclusion, ppp5K regulates inositol pyrophosphate metabolism in the vegetative stage but not its modulation during amoeba development (Fig. 3E).

We next assessed the isomeric nature of IP7 and IP8 in the ppp5k strain using 13C-NMR. After feeding ppp5k amoeba with 13C-inositol IP7 and IP8 were extracted and subjected to 13C-NMR analysis (Fig. 3F and G). The ppp5k-purified IP7 and IP8 spectra show the characteristic signature of pyrophosphate moiety at position 4/6 and 5 carbons. Like the wild type AX2 amoeba, the ppp5k strain possesses the 4/6-IP7 and 4/6,5-IP8 isomers. This is not surprising since PPIP5K are kinases thought to phosphorylate position one of the inositol ring (Lin et al., 2009; Wang et al., 2012); furthermore our vip1Δ rescue experiments (Fig. 2B and C) are also indicative of this specificity. While 13C-NMR sensitivity might fail to detect minor species of IP7 and of IP8 species, our theoretical consideration and our analysis suggest that amoeba Ppip5k, while not participating directly in the synthesis of the abundant 4/6,5-IP8, is nevertheless able to regulate its cellular levels.

3.5. Generation of the D. discoideum ip6k and ip6k-ppip5k strain

The inability to produce recombinant Ip6k and Ppip5k proteins to assess their biochemistry in vitro, prompted us to develop the full array of knockout strains to perform in vivo analyses. We re-generate the ip6k strain (see material and methods) isogenic to our AX2 background. Identically to the previously generated ip6k mutant, the new strain has no detectable level of IP8 and an almost completely depleted level of IP7 (Fig. 4A). Double mutants, in which both ppp5k and ip6k genes were disrupted, were generated starting from the ppp5k strain in which the blastocidin resistance gene (BSR) was excised by overexpressing a recombinant Cre (Faix et al., 2004). The ip6k gene was then disrupted to generate the ip6k-ppip5k strain (Fig. 4A). PAGE analysis of the ip6k-ppip5k reveals an inositol pyrophosphate profile similar to ip6k amoeba. Both the ip6k and the ip6k-ppip5k strains possess residual amounts of IP7 detectable by PAGE when extracts from 20 million cells were loaded on gel (Fig. 4A right panel), indicating the presence of an additional enzyme able to synthesize inositol pyrophosphates.

To verify the effect on the amoeba general fitness of the known inositol pyrophosphate synthesizing enzymes, we characterized the growth rate of AX2, ip6k, ppp5k, and ip6k-ppip5k amoebas (Fig. 4B). We did not observe major growth defects when the null strains were grown in rich HL5 synthetic media. Although we could observe the tendency for the mutant strains to grow slowly, this difference does not reach statistical significance. We next assessed the ability of the mutants to undergo starvation-induced development. All strains succeeded to follow development under standard KK2 agar conditions and form fruiting bodies (Fig. 4C). To detect any developmental phenotype that may have gone unnoticed in a phosphate rich buffer as KK2 (20 mM potassium phosphate buffer pH 6.8), the process of development of the double mutant ip6k-ppip5k was examined under complete phosphate starvation using TrisHCl or HEPES as buffer on cellulose filters. Despite variable differences in timing, not attributable to differing buffer conditions, ip6k-ppip5k completed development, culminating in the formation of fruiting bodies slightly smaller than the AX2 strain (Fig. 5).

3.6. CE-MS analysis of D. discoideum inositol pyrophosphate metabolism

To better elucidate the inositol pyrophosphate metabolism in the mutant strains, we next performed Capillary Electrophoresis Mass Spectrometry (CE-MS) analysis (Qiu et al., 2020). This sensitive analytical technique resolves with unprecedented resolution the different isomers of IP7 and IP8. CE-MS studies complement 13C-NMR analysis, which offers unique structural information, but lacks the degree of sensitivity of mass spectrometry detection.

The qualitative analysis of AX2 amoeba reveals 4/6-IP7 and 4/6,5-IP8 to be the major inositol pyrophosphates species, confirming the 13C-NMR studies (Fig. 6). However, two additional, IP7 isomers could be identified; 5-IP7 constituting about 20% of the entire IP7...
pools and 1-IP$_7$ representing roughly <5% of the entire IP$_7$ pools. As expected, the analysis of ppip5k strain reveals the absence of the minor 1/3-IP$_7$ species demonstrating that the *D. discoideum* Ppip5k likely phosphorylates the 1 position similarly to the mammalian counterpart (Wang et al., 2012) and as our yeast rescues experiment suggested (Fig. 2B and C). The Ppip5k synthesized 1/3-IP$_7$, while...
Fig. 5. Developmental progression of wild type AX2 and the double knockout ip6k-ppip5k strain. To assess if phosphate affects the developmental process of the ip6k-ppip5k strain, the AX2 and ip6k-ppip5k were developed on buffered cellulose filters. The buffers used were KK2 (potassium phosphate buffer), the phosphate-free HEPES buffer, and TRIS buffer. While developmental timings were inconstant between experiments, due to the variable amount of liquid in the imbedded filter, no consistent phenotype was detected.
not participating directly in the synthesis of the 4/6,5-IP$_8$, still regulates its cellular level (Figs. 3D and 4A).

Surprisingly, the analysis of ip6k mutant reveals the disappearance of the major IP$_7$ isomer the 4/6-IP$_7$ species (Fig. 6A). Thus, to the contrary of the mammalian IP6Ks D. discoideum homologous enzyme pyrophosphorylate position 4/6 of the inositol ring generating 4/6-IP$_7$. In light of these observations, in the ip6k-ppip5k amoeba, only the 5-IP$_7$ isomer could be detected. Therefore, D. discoideum must possess an additional kinase that we named Diphospho kinase 3 (Dpk3) responsible for 5-IP$_7$ synthesis and that together with the Ip6k would generate 4/6,5-IP$_8$. Fig. 6B summarises D. discoideum inositol pyrophosphate metabolism revealed by these analyses where Dpk3 represents an as-yet uncharacterised kinase capable of producing inositol pyrophosphates. The presence of three IP$_7$ isomers suggests the possibility for amoeba to synthesize three IP$_8$ species however only 4/6,5-IP$_8$ is detectable in our current CE-MS-qTOF experimental setup.

**Fig. 6.** CE-MS analysis of D. discoideum of AX2 and ip6k and ip6-ppip5k strains extracts. Qualitative CE-MS separation of TiO$_2$-purified D. discoideum extracts (A). Empty peak area indicates the migration of the indicated $^{13}$C$_6$-inositol standard while the filled peak area represents the elution of the amoeba extracted IP$_7$s (orange) and IP$_8$ (red). Enlarged inserts for the IP$_7$ region are presented to highlight the minor species of 1-IP$_7$ and 5-IP$_7$. This analysis reveals the absence of 1-IP$_7$ in ppip5 and of 4/6-IP$_7$ in ip6k stains and of both in ip6k-ppip5K. From this, we could deduce the inositol pyrophosphate pathway presented in (B) where Dpk3 stand for Diphosphate kinase number 3. This analysis was repeated three times giving identical results.
Fig. 7. *D. discoideum* polyP analysis of vegetative and development states of the inositol phosphate kinase mutants. Wild type AX2 and ip6k and ip6k-ppip5 strains growing exponentially in rich medium HL5 were collected (20 × 10^6 cells) or plated on KK2 agar plates for 16 h to stimulate developmentally induced polyP synthesis. The plates were scraped to recover *D. discoideum* and a neutralised acidic extract of the samples in vegetative stage (HL5) or after starvation (KK2) was prepared before loading on a 33% PAGE gel. The gels were stained with Toluidine blue to visualise inositol phosphate and polyP. While in this experimental set up polyP could not be detected in vegetative amoeba, polyP induction during development is clearly evident in both the wild type or mutant stains. OrangeG (OrG) is used as migrating dye. Developmental accumulation of polyP (B) is present in both ip6k (left panel) and in ppip5k (right panel) strains. Bromophenol Blue (BrB) is used as migrating dye. The figure is representative of experiments repeated at least three times.
3.7. polyP metabolism is not affected by D. discoideum Ip6k or Ppip5k

Based on our understanding of the link between inositol pyrophosphates and phosphate metabolism in yeast, the altered inositol pyrophosphates present in ip6k, ppip5k, and ip6k-ppip5k could influence directly or indirectly phosphate homeostasis in D. discoideum. The social amoeba possess sub-millimolar concentration of the phosphate-rich IP₆, IP₇, and IP₈ thus changing their concentration influences a large pool of cellular phosphate. Alternatively, inositol pyrophosphate could influence phosphate availability by regulating polyP metabolism, particularly as the primary function of polyP is to buffer cellular free phosphate concentration. We previously demonstrated that in S. cerevisiae polyP synthesis is under Kcs1 (the IP6K) control (Lonetti et al., 2011), while in S. pombe it is Asp1/Vip1 (the PPIPSK) that regulates polyP metabolism (Pascual-Ortiz et al., 2021). Therefore, in yeast, there is a clear link between inositol pyrophosphate and polyP cellular level even if the precise inositol phosphate kinase regulating polyP metabolism differs between yeast species.

The D. discoideum ip6k, ppip5k, and ip6k-ppip5k strains offer the opportunity to verify in an organism belonging to a different taxon if the synthesis of polyP is under control of Ip6K, of Ppip5k, of both or neither of the two enzymes. We extracted polyP using acidic phenol procedure from fast-dividing AX2, ip6k, ppip5k, and ip6k-ppip5k amoeba grown on rich HL5 medium and from KK2 agar plates for 16 h, a condition we previously demonstrated to induce polyP synthesis (Livermore et al., 2016). PAGE analysis of the extracted polyP revealed that while polyP is undetectable in this experimental setup from amoeba grown in HL5 medium, polyP under starvation conditions is detected in all four D. discoideum strains (Fig. 7A). We next followed the developmental synthesis and accumulation of polyP (Livermore et al., 2016). The ip6k and ppip5k amoebae were transferred on KK2 agar and cells were collected at different time points corresponding to the diverse developmental stages. PAGE analysis of phenol extract from ip6k and ppip5k amoebae revealed the dramatic accumulation of polyP during development as reported previously (Livermore et al., 2016). Therefore, in the social amoeba neither, the Ip6K or the Ppip5k are able to control polyP metabolism.

4. Conclusion

Studying the inositol pyrophosphate metabolism in ip6k, ppip6k and ip6k-ppip5k amoeba revealed interesting features. The amoeba Ip6k synthesizes 4/6-IP₇ instead of the 5-IP₇ isomer synthesized by its mammalian counterpart. Therefore, the definition of the inositol pyrophosphate species present in one specific organism cannot be extrapolated by sequence homology, but must be tested experimentally.

Conversely, the amoeba Ppip5k similarly to its mammalian counterpart, does pyro-phosphorylate position 1/3 of the inositol ring producing 1/3-IP₈, and therefore could not participate directly to the synthesis of the abundant 4/6,5-IP₈ isomer. The 1/3-IP₈ is by far the minor species of the three IP₇ isomer found in amoeba, but it does indirectly regulate IP₈ synthesis since ppip5k possesses a 50% decrease in 4/6,5-IP₈ level. These results indicate the existence of a third kinase, likely regulated by Ppip5k or its product, able to pyrophosphorylate position five, synthesizing 5-IP₇ and 4/6,5-IP₈. The recent discovery that Arabidopsis thaliana possesses three isomers of IP₇ and like in D. discoideum the most abundant is the 4/6-IP₇ species (Riemer et al., 2021), suggests the amoeba inositol pyrophosphates metabolism is conserved across many species.

Our work also reveals that in amoeba neither the Ip6k or the Ppip6k are involved in regulating polyP metabolism. This should not come as a surprise, since the synthesis of polyP in amoeba and yeast occurs using different enzymology. While yeast Vtc4 possesses an SPX domain that could be regulated by inositol pyrophosphates, the amoeba Ppk1 does not. Our work highlights how incorrect it is to extrapolate polyP yeast discoveries to other species when the mechanism of polyP synthesis is different as in amoeba or unknown as in mammals.

Surprisingly, ip6k, ppip6k and ip6k-ppip5k amoebas do not show major growth or developmental defects. We could not exclude that the minor species of IP₇ present in ip6k and the 5-IP₇ present in ip6k-ppip5k is sufficient to play signalling roles preventing the manifestation of inositol pyrophosphate-specific phenotypes. For this reason, it is imperative to identify the D. discoideum enzyme responsible to pyro-phosphorylate position five of the inositol ring Dpk3 (Fig. 6B) and thus responsible for the synthesis of the 5-IP₇ present in ip6k-ppip5k and for the synthesis of the abundant 4/6,5-IP₈ present in wild type amoeba. After identifying this additional kinase, the generation of the triple mutant strain might reveal the amoeba phenotypes associated with the absence of inositol pyrophosphates.

Credit author statement

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Declaration of competing interest

The authors declare none conflict of interest. The funding bodies do not have any role in the study design, and in data collection and analysis.

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Abbreviations

IP6  inositol hexakisphosphate, phytic acid  
IP7  diphosphinositol pentakisphosphate  
IP8  bis-diphosphinositol tetrakisphosphate  
IP6K  inositol hexakisphosphate kinase  
PPIP5K  diphosphoinositol pentakisphosphate kinase  
Vtc4  vacuolar transporter chaperone 4  
PPK1  polyphosphate Kinase 1  
polyP  inorganic polyphosphate  
SPX  protein domain named after SYG1/Pho81/XPR1  
AX2  axenic strains 2  
NMR  nuclear magnetic resonance  
CE-MS  capillary electrophoresis mass spectrometry  
qTOF  quadrupole time of flight  
BSR  blastocidin resistance gene  
PAGE  poly acrylamide gel electrophoresis  
OrG  Orange G  
BrB  bromophenol blue  
DpK3  diphospho kinase 3

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