Inositol Pyrophosphates and Their Unique Metabolic Complexity: Analysis by Gel Electrophoresis

Oriana Losito1, Zsolt Szijgyarto1, Adam Cain Resnick2, Adolfo Saiardi1*

1 Medical Research Council (MRC) Cell Biology Unit and Laboratory for Molecular Cell Biology, Department of Cell and Developmental Biology, University College London, London, United States of America, 2 Division of Neurosurgery at the Children’s Hospital of Philadelphia, Department of Neurosurgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, United States of America

Abstract

Background: Inositol pyrophosphates are a recently characterized cell signalling molecules responsible for the pyrophosphorylation of protein substrates. Though likely involved in a wide range of cellular functions, the study of inositol pyrophosphates has suffered from a lack of readily available methods for their analysis.

Principal Finding: We describe a novel, sensitive and rapid polyacrylamide gel electrophoresis (PAGE)-based method for the analysis of inositol pyrophosphates. Using 4',6-diamidino-2-phenylindole (DAPI) and Toluidine Blue we demonstrate the unequivocal detection of various inositol pyrophosphate species.

Conclusion: The use of the PAGE-based method reveals the likely underestimation of inositol pyrophosphates and their signalling contribution in cells when measured via traditional HPLC-based techniques. PAGE-based analyses also reveals the existence of a number of additional, previously uncharacterised pyrophosphorylated inositol reaction products, defining a more complex metabolism associated with the catalytically flexible kinase class responsible for the production of these highly energetic cell signalling molecules.

Introduction

Myo-inositol is a structurally simple sugar that has been exploited by evolution to generate a multitude of phosphorylated molecules with key signalling roles [1]. Inositol pentakisphosphate (IP₅) and phytic acid or inositol hexakisphosphate (IP₆) are the two most abundant inositol polyphosphates in mammalian cells. They are also the precursors of inositol pyrophosphate molecules that contain one or more pyrophosphate bonds [2,3]. Sequential phosphorylation of phytic acid gives rise to diposphoinositol pentakisphosphate (IP₇) and bisdiphosphoinositol tetrakisphosphate (IP₈) [4]. Likewise IP₅ is the precursor of additional inositol pyrophosphates such diposphoinositol tetakisphosphate (IP₉) and other less characterized pyrophosphate-containing molecules that retain an unphosphorylated ring hydroxyl [2,3]. Recently, an NMR study of pyrophosphate-containing inositols revealed that inositol pyrophosphate composition may indeed be more complex than previously realized, identifying the existence of a tri-phosphorylated species of “IP₉” or PPP-IP₅ [4].

Inositol pyrophosphates undergo rapid turnover in cells suggesting a potential signalling role for their metabolism [5]. Several studies have linked inositol pyrophosphates to disparate cellular functions from vesicular trafficking to telomere maintenance (for review see [2]). Their involvement in disease processes such as cancer and diabetes has also been suggested [6–9]. Given the higher free energy of hydrolysis possessed by the pyrophosphate moiety, soon after their initial discovery inositol pyrophosphates were suggested to participate in phosphotransferase reactions [10]. This hypothesis was verified [11]; recent work has demonstrated that IP₇ phosphorylates its substrates by donating its pyrophosphate β-phosphate moiety to pre-phosphorylated serine residues, generating a novel post-translational modification in the form of pyro-phosphorylated proteins [12].

Two distinct classes of evolutionarily conserved enzymes synthesize inositol pyrophosphates. The IP₆Ks posses extraordinary catalytic flexibility, pyrophosphorylating IP₆ and IP₅ respectively to PP-IP₄ and IP₇ and subsequently using these enzymatic products as substrates for the generation of more complex molecules containing two or more additional pyrophosphate moieties or a tri-phosphate species [4,13,14]. Recently, a second class of pyrophosphate generating enzymes was identified in yeast [15]. Initially, Vip1 was described as a specific inositol hexakisphosphate kinase able to convert IP₆ to IP₇ [15]. Further work has suggested that this protein can also sequentially convert IP₆ to IP₇ and IP₈. However, the kinetic parameters of the mammalian homolog (PPiPK5K or IP7K) indicate that this enzyme is likely to physiologically convert IP₇ to IP₈ [16,17] and thus might represent a previously identified IP7K activity [18].

The enzymatic conversion of IP₆ to IP₇ (IP₆-Kinase reactions) or IP₇ to IP₈ (IP₇-kinase reactions) are traditionally evaluated using...
a radiolabeled precursor such $^3$H-IP$_6$ or $^{32}$P-IP$_6$ which unfortunately are not commercially available. Mayer and colleagues have developed a chromatographic technique that utilizes a post-column modification of the phosphate groups to detect inositol polyphosphates using spectrophotometry [19]. However, this procedure is relatively insensitive and still requires separating the reaction products using sophisticated high-performance liquid chromatography (HPLC) apparatuses [19]. Here we describe a rapid, simple method for the analysis of highly phosphorylated inositol polyphosphates that takes advantage of the ease of polyacrylamide gel electrophoresis (PAGE) to resolve highly phosphorylated inositol polyphosphates combined with the use of 4',6-diamidino-2-phenylindole (DAPI) to uniquely visualize inositol polyphosphates. This simple and sensitive method allows for the reliable detection of nanomolar quantities of inositol polyphosphates. Furthermore our application of PAGE to the investigation of the enzymatic activities of IP$_6$K1 and Vip1 reveals an exceptionally robust inositol polyphosphate metabolism that has remained unidentified due to the inability of inositol polyphosphates using HPLC-based protocols.

Results

Gel electrophoresis analyses of inositol polyphosphates

Traditional methods used for inositol pyrophosphate analysis utilize high-performance liquid chromatography using a strong anion exchange column (SAX-HPLC) [20]. Alternatively, inositol polyphosphates can be analyzed using thin layer chromatography on polyethyleneimine cellulose (PEI-TLC) [13,21]. However, PEI-TLC lacks sufficient resolving power and is therefore a little used technique. Both chromatographic techniques require the use of custom made radioactive precursors. While gel electrophoresis is also commonly used to separate and study small molecules, it has yet to be applied to inositol polyphosphates. To evaluate its applicability, we tested the use of PAGE and commonly used phosphate stains to resolve and detect inositol polyphosphates. We ran 5-10 nmols of IP$_5$, IP$_6$, IP$_7$ and ATP on a 33.3% polyacrylamide gel and stained it with Toluidine Blue, a commonly used cationic metachromatic dye that binds phosphate groups (Figure 1A left). Phosphorylated inositols are easily resolved by gel electrophoresis with ATP migrating similarly to IP$_3$ (Figure 1A left). Recently a report identified the staining of inorganic polyphosphate (PolyP) chains by DAPI [22], a bivalent commonly used dye that stains phosphate-rich compounds such as DNA. An identical gel run in parallel was stained with DAPI (Figure 1A right). Exposure of the gel to UV revealed that IP$_5$, IP$_6$ and ATP display positive staining while the inositol pyrophosphate, IP$_7$, was negatively stained due to the photobleaching of DAPI. Similarly, the PolyP ladder used as an electrophoresis standard was also negatively stained, as reported [22]. It is noteworthy that the short PolyP forms are labeled by Toluidine Blue but remain unlabeled by DAPI, indicating that at least four polyphosphate moieties are required for negative staining by DAPI. Likely, the negative staining associated with IP$_7$ is due to the highly negative charge of the fully phosphorylated inositol ring that, with the addition of the pyrophosphate moiety, results in DAPI photobleaching. We have found that DAPI and Toluidine Blue stains can be sequentially performed on the same gel; however the Toluidine Blue staining looses sensitivity.

We evaluated the detection limits for these new staining methods resolving serial dilutions of IP$_3$ on polyacrylamide gels. Using Toluidine Blue, we were able to detect two nmols of IP$_7$ (data not shown). DAPI staining was much more sensitive allowing for the detection of less than 100 pmols of IP$_7$ (Figure 1B). Up to 100 nmols of the non-pyrophosphate containing IP$_5$ or IP$_6$ only stain positively by DAPI; however, quantities exceeding 200 nmols of IP$_7$ become negatively stained by DAPI (data not shown). This staining distinction between inositol pyrophosphates and their precursors provides unprecedented ease in the evaluation of IP$_7$-Kinase reactions.

Analysis of IP$_7$-Kinase reactions by PAGE

We incubated 2 nmols of IP$_7$ and trace amounts of $^3$H-IP$_6$ with recombinant mouse His-IP$_6$K1 and separated the reaction products by gel electrophoresis or by SAX-HPLC (Figure 2A,B). The reaction analysed by SAX-HPLC revealed the formation of radiolabeled IP$_7$ and IP$_8$ (Figure 2B). The equivalent enzymatic reaction separated by PAGE revealed the formation of two bands negatively labelled by DAPI (Figure 2A). These negatively stained bands migrated more slowly than IP$_6$ and their migration distance relative to that of the PolyP marker indicated that they likely possess seven and eight phosphate groups, respectively. To verify that the negatively stained bands correspond to IP$_7$ and IP$_8$ generated by IP$_6$K1, we cut 1 cm gel fragments and directly counted their radioactivity. This resulted in the recovery of only 20% of the input radioactivity (Figure 2A). However, dissolving the gel prior to scintillation counting resulted in 80-90% recovery of the input radioactivity (Table 1). In both cases the radioactivity initially contained in the positively stained bands of IP$_6$ was redistributed in the negatively stained bands following IP$_6$K1 enzymatic reactions, demonstrating that these bands correspond to the IP$_7$ and IP$_8$ reaction products (Figure 2A). These studies reveal that IP$_7$ is actually a relatively more abundant reaction product when analysed by PAGE (Table 1), suggesting that SAX-HPLC analyses might selectively degrade IP$_8$ (see below).

We performed a time course experiment incubating IP$_7$ and IP$_8$ with recombinant His-IP$_6$K1 (Figure 3A). Incubation for merely 10 min at 37°C already leads to the formation of two pyrophosphate species with both substrates. Using IP$_6$, as a substrate, the time course reveals the formation of at least five different inositol pyrophosphate species (Figure 3A and B). Because the structure of IP$_6$-derived inositol pyrophosphates may contain either a diphosphate or triphosphate species [4], we refer to the inositol pyrophosphates derived from IP$_6$ as PP-IP$_4$ to indicate bisdiphosphoinositol tetrakisphosphate and use 2P-IP$_3$, 3P-IP$_3$, etc. to indicate pyrophosphates derived from IP$_3$ containing seven, eight, or more phosphate groups. The fast migrating band generated from IP$_6$ migrates very close to that of IP$_7$, suggesting that this inositol pyrophosphate species likely contains seven phosphate groups (2P-IP$_7$) (Figure 3A) with PP-IP$_4$ remaining undetected by DAPI staining. To verify this assumption we increased the amount of IP$_6$, and IP$_7$ used in our enzymatic reactions to 10 nmols and ran two parallel gels (Figure 3B). Following a 10 min reaction, Toluidine Blue detected the presence of PP-IP$_4$ migrating as expected similarly to IP$_7$. Furthermore the increase in substrate concentration allowed PP-IP$_4$ to be also detected, albeit weakly, by DAPI (Figure 3B). Analysis of the relative intensities between the two staining methods revealed that different inositol pyrophosphate species have different DAPI photobleaching capacity. After a 10 min reaction, via Toluidine Blue PP-IP$_4$ was more intensely stained than 2P-IP$_7$, to the contrary DAPI displayed an opposite staining pattern (Figure 3B). Similarly, analysis of a 120 min reaction revealed that 3P-IP$_3$ is far more capable of photobleaching DAPI than 2P-IP$_3$. Parallel SAX-HPLC and PAGE analyses of His-IP$_6$K1 reactions using trace amounts of $^3$H-IP$_6$ and cold IP$_6$ as substrate, revealed that the more highly phosphorylated forms of inositol pyrophosphates are dramatically underrepresented by traditional HPLC-based chromatographic techniques (Table 1). These studies
indicate, as in the case of IP$_6$, that the SAX-HPLC method results in the selective degradation of some inositol pyrophosphate species (Table 1) (see below).

Time course analyses of IP$_6$K1 reactions using IP$_6$ as a substrate revealed the production of IP$_7$ and two additional, more phosphorylated species (Figure 3A); due to their close migration distance, it is likely that these bands represent two different forms of an inositol pyrophosphate containing eight phosphate groups “IP8”. One likely represents the recently described trisphosphate species PPP-IP$_5$ [4]. The analyses of IP$_6$K1 reactions run for longer times, such as overnight, using IP$_5$, IP$_6$ and IP$_7$ (isomer 5PP-IP$_5$ synthesized by IP$_6$K1) as substrates surprisingly revealed the production of novel, highly phosphorylated species. Using IP$_5$ as a substrate we observed the formation of large amounts of 4P-IP$_5$ and 5P-IP$_5$ products (Supporting Figure S1). However, only the most abundant and biologically relevant I(1,3,4,5,6)P$_5$ isomer is further phosphorylated to generate more complex inositol pyrophosphates (Supporting Figure S2). These data further demonstrates the catalytic flexibility of the IPK family of enzymes [23].

PAGE analysis allows for the first time for the evaluation of all six possible IP$_5$ isomers as substrates for IP$_6$K1. Incubation of the purified enzyme with each of the IP$_5$ isomers for two hours at 37°C revealed the ability of IP$_6$K1 to metabolize five of the isomers with only I(1,2,3,5,6)P$_5$ failing to convert to 2P-IP$_5$. However, only the most abundant and biologically relevant I(1,3,4,5,6)P$_5$ isomer is further phosphorylated to generate more complex inositol pyrophosphates (Supporting Figure S2). These data further demonstrates the catalytic flexibility of the IPK family of enzymes [23].

Figure 1. Separation and detection of inositol polyphosphates via PAGE. A) Analysis of 10 nmol of IP$_5$ and IP$_6$, 5 nmols of IP$_7$, and 100 nmol ATP resolved on a 33.3% PAGE and stained with either Toluidine Blue (left panel) or DAPI (right panel). Under UV exposure DAPI staining displays positive staining for ATP, IP$_5$, and IP$_6$, while the inositol pyrophosphate, IP$_7$, becomes negatively stained due to DAPI photobleaching. Similarly, the PolyP ladder used as an electrophoresis standard was also negatively stained. B) DAPI staining of serial dilutions of IP$_7$ resolved on 33.3% polyacrylamide gel reveals remarkable sensitivity, detecting <100 pmols.

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Recently, a new class of pyrophosphorylating enzymes was discovered consisting of kinases which are also capable of sequentially converting IP₆ to IP₇ to IP₈. The yeast protein, Vip1, was originally described as an IP₆-Kinase responsible of converting IP₆ to IP₇ [15]. However, the mammalian homolog (PP-IP₅K) has kinetic characteristics suggesting it may represent a physiological IP₇-Kinase (IP₇K) [16,17]. Initially, we performed a time course experiment incubating IP₆ with recombinant yeast Vip1 (Supporting Figure S3). Incubation for merely 5 min at 37°C already leads to the formation of two pyrophosphate species with the formation of at least four species after two hours of incubation (Supporting Figure S3). We then decided to examine the enzymatic activity of Vip1 via gel electrophoresis in three parallel reactions using IP₅, IP₆, or IP₇ as a substrate. Incubation of recombinant Vip1 with the three different substrates for two hours at 37°C revealed the inability of this enzyme to metabolize IP₅, as previously reported [16] (Figure 4A). When IP₆ is used as a substrate, it is rapidly converted to IP₇, IP₈, and a further phosphorylated form containing nine phosphate groups. When IP₇ (isomer 5PP-IP₅ synthesized by IP₆K₁) is used as a substrate, an IP₈ species is formed that migrates slightly slower than that...
generated by the sequential phosphorylation of IP₅ to IP₉, suggesting a different isomer. Because Vip1 was described to pyrophosphorylate the racemic 1 and 3 ring positions [24], conceivably the IP₅ generated from IP₆ represents the (1,3)PP-IP₅ isomer while the ((1 or 3),5)PP-IP₅ is the isomer generated using 5PP-IP₅ as a substrate. Using 5PP-IP₅ as a substrate, two further phosphorylated inositol species were detected (Figure 4A) with their relative migration distance suggesting they represent two different IP₅ isomers. One of the IP₅ species co-migrates with IP₅ generated using IP₆ as a substrate (Figure 4A). We tested if the inositol pyrophosphate products generated by IP₆K₁ and Vip1 can be reciprocally exchanged as substrates (Figure 4B). The parallel analysis of IP₆K₁ and Vip1 reactions using IP₆ as a substrate revealed slightly different IP₅ and IP₆ migration rates (Figure 4A and B), indicating that the two enzymes generate different inositol pyrophosphate isoforms as recently demonstrated [4,24]. However, adding Vip1 to the IP₆K₁ reaction or adding IP₆K₁ to Vip1 reactions generates similar inositol pyrophosphate products with the abundant formation of IP₆ and a robust synthesis of more phosphorylated IP₃ inositol pyrophosphate species (Figure 4B). Similarly, we tested Vip1 activity towards the inositol pyrophosphates generated by IP₆K₁ using IP₆ as a substrate (Figure 4C); IP₅ was first incubated with IP₆K₁ and Vip1 was subsequently added to the assay. This result occurred in the vigorous production of 2P-IP₅ and 3P-IP₅ species indicating that PP-IP₅ and perhaps other inositol pyrophosphates generated from IP₆ by IP₆K₁ are genuine enzymatic substrates of Vip1. These conclusions have been confirmed in vivo by our analysis of the inositol polyphosphate profile of the ipk1Avip1A double mutant than shows a massive accumulation of PP-IP₅ (Supporting Figure S4) suggesting that the Vip1 catalytic activity is more promiscuous than previously described.

**Inositol pyrophosphates are sensitive to acidic degradation**

The parallel analyses by SAX-HPLC and PAGE of IP₆K₁ enzymatic products revealed a consistent under-representation of highly phosphorylated inositol pyrophosphate species in the chromatographic studies (Table 1). One of the main differences between SAX-HPLC and PAGE analyses is the requirement in the former for acidic buffer (pH 3.8) running conditions and the deproteinization of the sample, typically using high concentrations of perchloric acid. In contrast, for PAGE analysis the samples are unprocessed and loaded directly into the gel at pH 8.0. To test if acidic conditions may be responsible for inositol pyrophosphate degradation, we incubated IP₆K₁ reactions for 30 min at room temperature or at 37°C in 1 M perchloric acid before neutralizing with potassium carbonate (Figure 5A). Surprisingly, we observed a dramatic degradation of IP₅, derived inositol pyrophosphates and a substantial degradation of IP₆, with the complete disappearance of the faster migrating species (Figure 5A). Similarly, Vip1-generated inositol pyrophosphates were also sensitive to acidic conditions. We observed the disappearance of the IP₅ band and substantially decreased DAPI staining when the sample was incubated on ice (Figure 5B); further degradation was observed when the sample was incubated at room temperature while at 37°C only no inositol pyrophosphates were observed with the resultant generation of IP₉ detected by Toluidine Blue staining (Figure 5B).

**Discussion**

Inositol pyrophosphates are attracting increased attention for their possible role as a signalling molecule and have been linked to a wide range of biological functions, including vesicular trafficking, apoptosis, DNA repair, telomere maintenance, and stress responses [2]. Similarly, important human diseases such cancer and diabetes appear to be under inositol pyrophosphate control [6–9]. However, significantly more research is necessary for the elucidation of the full spectrum of physiological mechanisms

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**Table 1. Comparison of inositol pyrophosphate recovery between SAX-HPLC and PAGE technology.**

<table>
<thead>
<tr>
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<th>SAX-HPLC (CPM)</th>
<th>%</th>
<th>PAGE (CPM)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP₀</td>
<td>1100(+/−345)</td>
<td>6.0</td>
<td>309(+/−155)</td>
<td>1.7</td>
</tr>
<tr>
<td>IP₁</td>
<td>14623(+−976)</td>
<td>79.5</td>
<td>11078(+−578)</td>
<td>63.3</td>
</tr>
<tr>
<td>IP₂</td>
<td>2680(+−567)</td>
<td>14.6</td>
<td>6098(+−897)</td>
<td>34.9</td>
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Parallel kinase reactions containing IP₆K₁ and 2 nmol of IP₆ or 2 nmol of IP₈ and trace amounts of ¹³H-IP₅ or ¹³H-IP₆ were run for two hours at 37°C. One set of reaction products were analyzed by SAX-HPLC after incubating the samples for 20 minutes with perchloric acid (1 M) on ice to precipitate the proteins. The second set of reactions were resolved by PAGE and subjected to DAPI staining, the bands corresponding to inositol polynucleotide species were then cut and subjected to scintillation counting. The data (CPM) represent the averages+/− standard deviation of three independent experiments. The percent values represent the proportional ratio of the inositol polynucleotide species recovered.

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controlled by this class of signalling molecules. Unfortunately, the current state of the art experimental techniques used to analyze inositol pyrophosphate metabolism require sophisticated apparatuses such as an HPLC machine as well as the ability to synthesize and purify radiolabeled precursors. In contrast, PAGE separation simply requires a universally available gel electrophoresis apparatus. Furthermore, we’ve identified DAPI as a reliable and very sensitive stain that allows for the detection of the pyrophosphate moiety. The combination of PAGE and DAPI staining represents a rapid, easy and widely available method to the evaluation of inositol pyrophosphates. Here, we used this method to study IP6K1 and Vip1 enzymatic reactions, revealing the existence of a number of additional, previously uncharacterised pyrophosphorylated inositol species. More importantly, parallel analyses comparing SAX-HPLC- and PAGE-based methods reveal a significant underestimation of the quantity and composition of inositol

Figure 3. IP6K1 displays catalytic flexibility and multiple reaction products using both IP₅ and IP₆ as substrates. Kinase reactions were performed with recombinant IP6K1 using 2 nmols of IP₅ or IP₆ as substrate. (A) Time course analyses of reaction products using either IP₅ or IP₆ as substrate reveal multiple reaction products. (B) Increasing substrate concentrations to 10 nmols allows for the detection of PP-IP₄ upon DAPI staining. Results demonstrate the differential ability of different inositol pyrophosphate to induce DAPI photobleaching.

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Inositol pyrophosphates are typically subjected to acidic conditions before and during SAX-HPLC analysis. The study of these treatments using PAGE revealed the degrading effects of such actions.

The measurement of inositol pyrophosphates extracted from cells would represent the next step forward in the application of the new PAGE technology. Unfortunately, our current efforts have failed to optimize a successful protocol (Supporting Figure S5).

Perchloric acid treatment, commonly used for inositol polyphosphate extraction of yeast cells [20], mainly extracts inorganic polyphosphates that are very abundant in this organism [26] and are negatively stained by DAPI [22]. The co-purification of PolyPs obscures the inositol pyrophosphates present in the cell extract, making them unidentifiable (Supporting Figure S5). We are currently developing enzymatic strategies to remove co-purifying molecules to allow for the measurement of inositol pyrophosphates extracted from cells. Furthermore, to fully appreciate the complexity of inositol pyrophosphate metabolism.

**Figure 4. Comparison of Vip1 and IP6K1 inositol pyrophosphorylation activities.** Kinase reactions were performed with recombinant IP6K1 and Vip1 using 2 nmols of IP₅ or IP₆ and IP₇ as substrate for two hrs at 37°C. A) Vip1 and IP6K1 possess differing substrate specificities and capacities for the sequential phosphorylation of inositol pyrophosphates. B) Vip1 and IP6K can function sequentially to generate IP₈ and IP₉ using IP₆ as a substrate. Toulidine Blue staining (bottom) was performed sequentially following DAPI staining. C) Vip1 can function to sequentially phosphorylate IP₆K1 reaction products generated using IP₅ as the initial substrate. Toulidine Blue staining (left) was performed sequentially following DAPI staining.

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Consequently only the development of new, milder extraction techniques will allow for the full appreciation of the metabolic complexity of the inositol pyrophosphates.

Our identification of DAPI staining’s ability to differentiate between inositol pyrophosphates and their precursor provides a useful tool for the rapid analysis of in vitro IP6-6,7,8-Kinase reactions. The evident degradation of inositol pyrophosphates under the acidic conditions traditionally used for their analysis suggests alternate methods must be developed for their in vivo evaluation as well. DAPI staining may indeed provide such a technique. DAPI is widely used as a DNA stain for fluorescence microscopy, emitting in the blue spectrum at 456 nm. DAPI has also been used to label cellular pools of PolyP, where it emits in the yellow spectrum at 540 nm [27]. We are currently evaluating the fluorescence emission spectrum of DAPI and related molecules when bound to various inositol pyrophosphates. Development of such a labelling method may ultimately permit for the evaluation of the dynamic metabolism of inositol pyrophosphates in intact cells.

Finally, the availability of a rapid method for analyzing the IP6Ks/Vip1s reactions allows for the identification of small molecule inhibitors or enhancers using a small chemical compound library. Conversion of IP6 to higher inositol pyrophosphates can be easily analysed on small 10×6 cm gels (data not shown) that can be prepared, run, and stained in less than two hours, allowing for the simultaneous analysis of 100 s of reactions. Using traditional HPLC-based assays such analyses would be entirely impractical. The potential therapeutic potential of such compounds is supported by the recent identification of the critical role inositol pyrophosphates play in insulin secretion and oncogenic processes [6–9]. Though the inositol pyrophosphate field appears to be more complex than previously described, our identification of a PAGE-based analytic method will serve to increase both access to and the ease with which we can study these highly energetic cell signalling molecules.

**Methods**

**Reagents**

Polyacrylamide mix, TEMED, ammonium persulfate, the gel solubilizer Solucint-O were acquired from National Diagnostic; all others reagents were purchased from the Sigma-Aldrich Company. A second source of phytic acid was purchased from Calbiochem. Tritium [3H]-IP5 and [3H]-IP6 were purified from mutant yeast respectively, as previously described [20,25]. IP5 was synthesized with IP6K1 and purified as previously described [11,20]. The plasmids expressing His-IP6K1 (mouse), and GST-Vip1 (yeast, kinase domain) and the procedures for purifying the respective recombinant enzymes were previously described [12,28].

**Inositol polyphosphates kinase reactions**

The reaction mix contained: 2 µl 5× Buffer (150 mM Hepes 6.8; 250 mM NaCl; 30 mM MgSO4; 5 mM DTT; 5 mM NaF); 0.5 µl phosphocreatine (200 mM); 0.5 µl creatine phosphokinase (800 U/µl); 0.5 µl ATP-Mg (10 mM); 2–10 nmol IP5/IP6/IP7; and 5 to 30 ng of the appropriate enzyme. Trace amounts of [3H]-IP5 or [3H]-IP6 (~20,000 CPMs) was added when indicated. The reactions were incubated at 37°C for the indicated times. Reactions were then stopped by the addition of 2 µl EDTA (100 mM) and placed on ice. The samples were then run on a polyacrylamide gel, frozen at ~20°C, or processed for SAX-HPLC analysis as previously described [25]. Briefly, 50 µl of 1 M perchloric acid was added to the samples followed by the addition of 25–30 µl of 1 M potassium carbonate containing 3 mM EDTA to neutralize the mixture.

**Fractionation of inositol polyphosphates by PAGE**

Inositol polyphosphates were resolved using 24×16×0.1 cm gel using 33.3% polyacrylamide gel in TBE (31.7 ml 40% Act/Bis (19:1); 3.8 ml 10× TBE; 2.2 ml H2O; 270 µl 10% APS; 30 µl TEMED). Gels were pre-run for 20 minutes at 300 volts. Then 5–10 µl of 6× Dye (10 mM TrisHCl pH 7.0; 1 mM EDTA; 30% glycerol; 0.1% Orange G) was added to each sample prior to loading onto gels. Gels were run at 300–400 volts overnight at 4°C.
until the Orange G dye front reached 10 cm from the gel's bottom. To analyse in gel radioactivity distribution, serial, one cm gel fragments were cut after DAPI staining over a UV transilluminator. Gel fragments were incubated overnight with 1–2 ml of the gel solubilizer SoluSol; 15–20 ml of Solucint-O cocktail was added and radioactivity was assessed with a β-counter.

**Toluidine Blue staining**

Gels were gently agitated for 30 min at room temperature in a filtered staining solution (20% methanol; 2% glycerol; 0.05% Toulidine Blue), then destained for 5 min with several changes of the same solution without dye. Pictures were taken after exposing the gel on a white light transilluminator.

**DAPI staining**

Gels were gently agitated for 30 min at room temperature in staining solution (20% methanol; 2% glycerol; 20 mM Tris base; 2 μg/ml DAPI). Gels were destained for 45 min in the same solution without DAPI and exposed at 365 nm over a UV transilluminator for 2–10 min to induce photobleaching, after which photographs were taken.

**Supporting Information**

**Figure S1** IP6K1 displays the ability to synthesize IP13 in vitro. Found at: doi:10.1371/journal.pone.0005580.s001 (0.24 MB PDF)

Figure S2 IP6K1 displays a wide range of IP5 isomeric substrate specificities. Found at: doi:10.1371/journal.pone.0005580.s002 (0.20 MB PDF)

Figure S3 Time course analyses of VIP1 reaction products. Found at: doi:10.1371/journal.pone.0005580.s003 (0.03 MB PDF)

Figure S4 Inositol polyphosphate profile of ipk1Δ and ipk1Δ-vip1Δ. Found at: doi:10.1371/journal.pone.0005580.s004 (0.12 MB PDF)

Figure S5 PAGE analysis of inositol polyphosphates extracted from wild type and kcs1Δ yeast. Found at: doi:10.1371/journal.pone.0005580.s005 (0.16 MB PDF)

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**Author Contributions**

Conceived and designed the experiments: OL ACR AS. Performed the experiments: OL ZS AS. Analyzed the data: OL ZS AS. Contributed reagents/materials/analysis tools: ACR AS. Wrote the paper: ACR AS.

**References**