Biochem. J. (1998) **335**, 671–679 (Printed in Great Britain)

Inositol hexakisphosphate in *Schizosaccharomyces pombe*: synthesis from Ins(1,4,5)P_3 and osmotic regulation

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Schizosaccharomyces pombe extracts synthesize InsP_6 (*myo*-inositol hexaphosphate) from Ins(1,4,5)P_3 plus ATP. An *S. pombe* soluble fraction converts Ins(1,4,5)P_3 into Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4, in a constant ratio of $\approx 5:1$, and thence to Ins(1,3,4,5,6)P_5 and InsP_6. We have purified a soluble Mg^{2+}-dependent kinase of molecular mass ≈ 41 kDa that makes Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 in the same ratio and also converts Ins(1,4,5,6)P_4 or Ins(1,3,4,5)P_4 into Ins(1,3,4,5,6)P_5 and InsP_6. Of InsP_3 isomers other than Ins(1,4,5)P_3, only the non-biological molecule Ins(1,4,6)P_3 potently 'competed' with all steps in conversion of Ins(1,4,5)P_3 into InsP_6. Examination of molecular graphics representations allowed us to draw tentative conclusions about the environment needed for an hydroxyl group to be phosphorylated by this kinase and to predict successfully that the purified kinase would phosphorylate the 5-hydroxyl of Ins(1,4,6)P_3. *S. pombe* that have been cultured with [^{3}H]inositol contains a variety of ^{3}H-labelled inositol polyphosphates, with Ins(1,4,5)P_3 and InsP_6 the most prominent, and the InsP_6 concentration quickly increases in hyper-osmotically stressed *S. pombe*. This yeast therefore contains InsP_6 and Ins(1,4,5)P_3 as normal constituents, makes more InsP_6 when hyper-osmotically stressed and contains a versatile inositol polyphosphate kinase that synthesizes InsP_6 from Ins(1,4,5)P_3.

INTRODUCTION

Since phosphatidylinositol (PtdIns) (4,5)P_2 hydrolysis to *myo*-inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] and diacylglycerol was recognized as a widespread eukaryotic signalling pathway [1–5], *myo*-inositol derivatives have been assigned several other cellular roles [6]. Additional signalling pathways involve receptor-stimulated PtdIns(3,4,5)P_3 synthesis from PtdIns(4,5)P_2 by phosphoinositide 3-kinases [7,8] and PtdIns(3,5)P_2 synthesis by a stress-activated PtdIns3P 5-kinase [9,10]. Polyphosphorylated inositol glycerolipids and inositol polyphosphates have also been increasingly implicated in several other central cell functions (see [6,11–15]).

Many elements of these signalling pathways, though not PtdIns(4,5)P_2 3-kinases, are found in yeasts (e.g. *Neurospora crassa*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*), in which inositol utilization has been analysed extensively [16–26]. The stress-regulated PtdIns(3,5)P_2 pathway was described in [10], and yeast inositol lipids and/or phosphates may have roles in cell cycle control and responses to nutrient and osmotic stresses and mating pheromones [22–26]. It is still not clear whether yeast Ins(1,4,5)P_3 is made by the one phosphoinositidase C present in these organisms (homologous to mammalian phosphoinositidase C-δ [16–19]) or by some other route, and whether it has a regulatory role. Although it has been suggested that Ins(1,4,5)P_3 provokes Ca^{2+} release from yeast vacuole preparations [20,21], the *S. cerevisiae* genome lacks a recognizable Ins(1,4,5)P_3-sensitive receptor or Ca^{2+} channel, so Ins(1,4,5)P_3 seems unlikely to regulate intracellular [Ca^{2+}] in yeasts in the same manner as in animal cells [27,28]. Disruption of the PtdIns(4,5)P_2 5-phosphatase genes causes aberrant vacuole formation, plasma membrane morphology, osmotic sensitivity and growth [29–31].

It is also not clear what roles the other water-soluble inositol polyphosphates present in yeasts play in their physiology. Eukaryote cells (including fungi) contain many inositol polyphosphates other than Ins(1,4,5)P_3, mostly of undetermined function, with InsP_6 usually the most abundant. Depending on the organism, InsP_6 can be made by receptor-independent pathways or from receptor-generated Ins(1,4,5)P_3, via Ins(1,3,4,5)P_4, Ins(1,3,4)P_3 and Ins(1,3,4,6)P_4 [6,11,12,32]. *Candida albicans* extracts convert Ins(1,4,5)P_3 into an unidentified InsP_4 and to InsP_5 and/or InsP_6 [24]. Phosphorylation, rather than dephosphorylation, seems to be the predominant fate of Ins(1,4,5)P_3 in *S. cerevisiae* [33,34] and *S. pombe* (this study). Both *S. cerevisiae* and *S. pombe* have an Ins(1,4,5)P_3 6-kinase [33,35], which in plants is a step in InsP_6 synthesis [36].

In this paper, we show that the concentration of InsP_6 in *S. pombe* is regulated by stress, and that the Ins(1,4,5)P_3 6-kinase of this yeast is a multi-functional kinase that phosphorylates Ins(1,4,5)P_3 to both Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4, and also converts these InsP_4s, via Ins(1,3,4,5,6)P_5, into InsP_6.

MATERIALS AND METHODS

Materials

Du Pont-New England Nuclear Corp. supplied [^{3}H]inositol, [^{3}H]inositol phosphates and [γ-^{32}P]ATP. *S. pombe* strains came from Dr. J. Davey, University of Warwick, Coventry, U.K. (h^- $sxa2^-$) and Professor P. Russell, Scripps Research Institute, La Jolla, CA, U.S.A. (R109). Ins(1,2,3)P_3, Ins(1,2,4)P_3, Ins(1,2,5)P_3, Ins(1,2,6)P_3, Ins(1,3,4)P_3, Ins(1,3,5)P_3, Ins(1,4,5)P_3, Ins(1,4,6)P_3, Ins(1,5,6)P_3, Ins(2,4,5)P_3, Ins(2,4,6)P_3 and Ins(4,5,6)P_3 were gifts from Dr. S.-K. Chung (POSTECH, Korea). Pharmacia supplied S-Sepharose, HR 75–Sepharose, chelating-Sepharose, NAP-10

Abbreviations used: Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4,5,6)P_5, InsP_6 etc. represent *myo*-inositol polyphosphates, numbered by reference to D-*myo*-inositol 1-phosphate as Ins1P; PtdIns, phosphatidylinositol; TEAB, triethylammonium hydrogen carbonate; FA, formic acid; AF, ammonium formate.

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and SR 75 gel-filtration columns. Centriprep-10 concentrators were from Amicon, and AG-1 resin (× 8, 200–400 mesh, formate form) and Silver Stain Plus kit were from Bio-Rad. Whatman supplied Partisil 10-SAX and Partisphere 5-SAX columns, scintillation fluids (Ultima Flo AF and AP) were from Canberra Packard, and triethylammonium hydrogen carbonate (TEAB) was from Fluka Biochemie. Sigma supplied most other reagents.

Preparation of yeast soluble fraction

S. pombe ($h^{-}sxa^{2-}$ strain), grown to mid-log phase (4×10^{6} cells/ml) in 1 litre of Dulbecco's modified Eagle's medium, were harvested, washed with 0.9 % NaCl and resuspended in a buffer containing 20 mM Hepes, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 μg/ml of leupeptin and 10 mM benzamidine. These and subsequent steps were at 4 °C. Yeasts were lysed by vortexing with glass beads 5 times for 1 min, with 1 min intervals. Centrifugation at 15000 ***g*** (15 min) and then 100000 ***g*** (1 h) yielded a 'soluble fraction'. The 100000 ***g*** pellet was washed once and resuspended, yielding a 'particulate fraction'.

Inositol phosphate kinase incubation conditions

Most experiments used [^{3}H]Ins(1,4,5)P_3 [1–5 nM; either 20000 d.p.m. (for analysis of products by Dowex mini-column chromatography) or 100000 d.p.m. (for analysis on HPLC anion-exchange column)] or other ^{3}H-labelled inositol polyphosphates as substrate, under first-order conditions, with 5 mM ATP as phosphate donor. Assays were also undertaken with unlabelled inositol phosphates and [γ-^{32}P]ATP (0.5 μM in 0.1 ml assays containing $\approx 5\times10^{5}$ d.p.m. [γ-^{32}P]ATP, purified by HPLC immediately before use). Yeast cell fractions or purified kinase preparations were incubated at pH 7.5 and 28 °C in 20 mM Hepes/1 mM EGTA containing ATP (for concentrations, see above), 10 mM creatine phosphate and 10 units/ml creatine kinase. ^{3}H-Labelled assays were stopped with 10 % (w/v) perchloric acid, left for 10 min on ice and centrifuged (15000 ***g***, 5 min). After neutralizing with 20 mM Hepes/KOH (pH 7.2) and standing for 30 min on ice, precipitated salt was removed by centrifugation. The assays with [γ^{32}P]ATP lacked creatine phosphate and creatine kinase, and incubations (for 2 h at 28 °C) were terminated with 1 ml ice-cold water and deproteinized on an NAP10 desalting column (Pharmacia). The eluate from the [^{32}P]ATP assays were spiked with [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,4,5,6)P_4 and analysed by HPLC on a Partisil 10 SAX column, using the pH 3.8 $(NH_4)_2PO_4$ gradient described below. This gradient separates the InsP_4 isomers from closely eluting contaminants in the [γ-^{32}P]ATP better than the pH 4.4 gradient used for other experiments.

Labelling of yeast with [^{3}H]inositol, and analysis of water-soluble yeast metabolites and products of enzyme assays

S. pombe PR109 (*h⁻leu⁻32ura4⁻D18*) were labelled by growing in YEAL media (0.5 % yeast extract, 250 mg/l each of adenine, leucine and uracil) with 3 μCi/ml [^{3}H]inositol for 30 h, harvested and washed. Chloroform/methanol/conc. HCl (3.75 vols; 200:400:5, by vol.) was added to 1.5 ml of cell suspension, vortexed, frozen in liquid N_2 and thawed. Phases were separated by adding chloroform (1.25 vols) and 0.1 M HCl (1.25 vols) and centrifuging. The ^{3}H-labelled water-soluble metabolites in the upper phase were analysed on Dowex mini-columns or by HPLC.

Inositol phosphate classes were separated on 6 × 25 mm AG-1 (× 8, 200–400 mesh, formate) columns; samples were diluted 10-fold before loading. They were eluted with 0.18 M ammonium formate (AF)/0.1 M formic acid (FA) (for Ins*P*s species); 0.4 M AF/0.1 M FA (InsP_2s); 0.8 M AF/0.1 M FA (InsP_3s); 1.2 M AF/0.1 M FA (InsP_4s) and 2 M AF/0.1 M FA (InsP_5s + InsP_6). Each fraction was mixed with 3 ml of 5 M AF and 10 ml of scintillant and radioactivity was measured.

Before individual inositol polyphosphate species were separated by anion-exchange HPLC, samples were neutralized with 20 mM Hepes (pH 7.4). A 235 × 4.6 mm Partisphere 5-SAX column was eluted with a phosphate gradient at 1 ml/min. Buffer A was water and buffer B 1.25 M diammonium orthophosphate, pH 4.4 (or, occasionally, pH 3.8, where indicated). The gradient was: 0–15 min, 0 % B; 46 min, 6 % B; 50 min, 15 % B; 90 min, 22 % B; 95 min, 38 % B; 150 min, 46 % B; 180–189 min, 100 % B; 190–200 min, 0 % B. The eluate was either collected (0.25 ml/fraction) or continuously mixed with scintillant (3 ml/min) and fed into an on-line scintillation detector (0.1 min averaging periods).

Preparation of [^{3}H]Ins(1,4,5,6)P_4 and [^{3}H]Ins(1,3,4,5,6)P_5

[^{3}H]Ins(1,4,5,6)P_4 was made by phosphorylating [^{3}H]Ins(1,4,5)P_3 with partially purified *S. pombe* Ins(1,4,5)P_3 kinase. [^{3}H]Ins-(1,3,4,5,6)P_5 was made by incubating [^{3}H]Ins(1,3,4)P_3 with rat liver cytosol [32]. They were purified by HPLC, and peak fractions were pooled, neutralized with 20 mM Hepes/KOH (pH 7.2), diluted 10-fold, and loaded on to 200 μl AG-1 (formate form) resin columns. These were washed with 10 ml of 0.3 M AF/0.1 M FA and 10 ml of water. TEAB (10 ml of 1 M) then eluted Ins(1,4,5,6)P_4, and 20 ml of 1 M TEAB eluted Ins-(1,3,4,5,6)P_5: TEAB was removed by lyophilization.

Purification of the inositol polyphosphate kinase

All steps were at 4 °C and pH 7.5. Yeast soluble fraction, from a 20 litre culture, was introduced at 4 ml/min into a 50 ml Mono S cation-exchange column (6 × 2 cm) pre-equilibrated with buffer A (20 mM Hepes/5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA). The column was washed with 100 ml of buffer A, followed by a gradient of 0–0.5 M KCl (250 ml): peak activity eluted at 0.4 M KCl. Pooled active fractions were passed at 1 ml/min through a 20 ml immobilized zinc column (2 × 12 cm) pre-equilibrated with buffer C (20 mM Hepes/0.5 M NaCl). The column was washed with 80 ml of buffer C, and kinase activity was eluted with 30 mM imidazole in buffer C. Active fractions were pooled, concentrated to 10 ml (Centriprep-10) and dialysed against 5 litres of buffer A overnight. The dialysate was passed at 1 ml/min through a heparin–Sepharose column (2 × 12 cm), which was washed with 40 ml of buffer A, followed by gradients of 0–0.5 M KCl and 0.5–1 M KCl: the kinase eluted at 0.45 M KCl. Peak fractions were concentrated to 0.5 ml and introduced into an HR75–Sepharose column pre-equilibrated and eluted with buffer D (20 mM Hepes/5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/2 % glycerol/0.15 M NaCl; 0.25 ml fractions). This kinase preparation was stored at −70 °C.

SDS/PAGE

Enzyme preparations in 62 mM Tris/HCl (pH 6.8)/3 % SDS/5 % 2-mercaptoethanol/10 % glycerol were separated on a 12 % polyacrylamide gel at 100 mV. The gel was silver stained by Bio-Rad protein staining kit.

Energy-minimized molecular models of inositol phosphates

The molecular structures of the relevant inositol phosphates were imported into QUANTA, and these structures were subjected to

simulated heating and equilibration in the CHARMm molecular dynamics programme, to allow the molecules to assume minimum-energy conformations.

RESULTS

S. pombe contains a variety of inositol polyphosphates

Little is known of the normal inositol polyphosphate complement of yeasts. Figure 1 shows a profile of the labelled inositol derivatives with two or more phosphate groups that are found in *S. pombe* that had been labelled to close to isotopic equilibrium with [^{3}H]inositol: less highly charged peaks were not analysed in detail. The cells were grown to stationary phase with [2-^{3}H]inositol, extracted, inositol phosphates separated by high-resolution anion-exchange HPLC and radioactivity determined in 0.5 min fractions. The most prominent peaks eluted in positions characteristic of InsP_6 (also known as phytate), Ins(1,4,5)P_3 and three InsP_2 isomers. Smaller peaks had the elution characteristics of other InsP_3 isomers, two InsP_4 isomers [eluting as Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4] and an InsP_5 [co-eluting with Ins(1,3,4,5,6)P_5]. Extracts from exponentially growing cells contained similar compounds, though mainly at lower concentrations (results not shown).

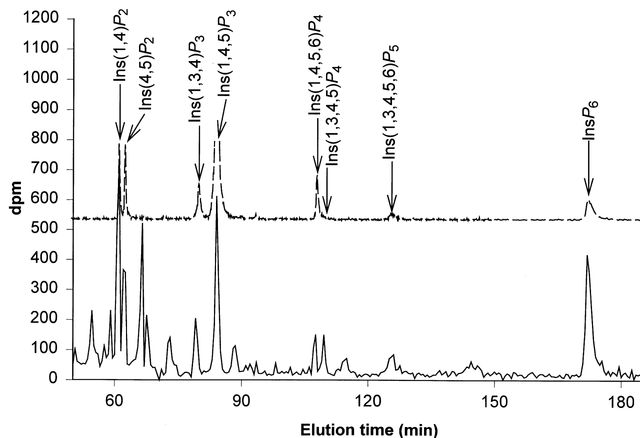


Figure 1 Anion-exchange HPLC analysis of ^{3}H-labelled inositol metabolites in *S. pombe* labelled to equilibrium during growth to stationary phase

The upper (broken) trace defines the elution positions of a number of common inositol polyphosphates. The lower (solid) trace shows the *S. pombe* inositol metabolites: 0.5 min fractions were collected and their radioactivity determined. The displayed profile is typical of those seen in 3 experiments.

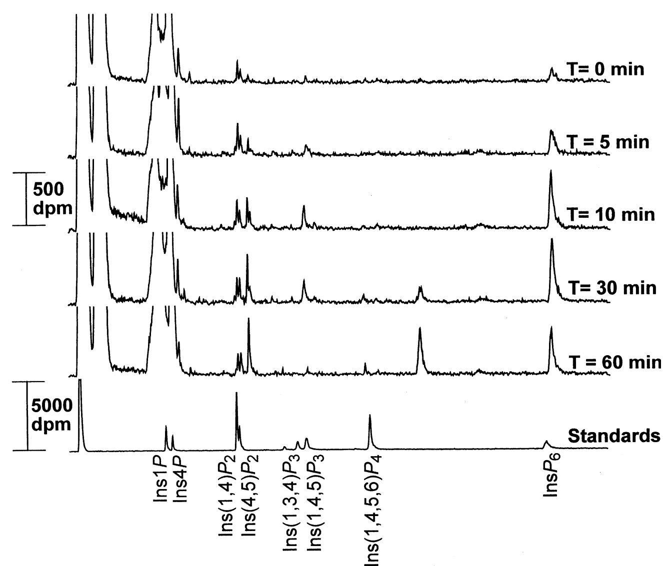


Figure 2 Hyper-osmotic stress stimulates InsP_6 synthesis in *S. pombe*

HPLC separation of the ^{3}H-labelled inositol metabolites extracted from [^{3}H]inositol-labelled stationary-phase cells that were either unperturbed or subjected to hyper-osmotic treatment with 0.7 M KCl for the times (T) indicated. Radioactivity eluting from the HPLC column was detected using an on-line liquid-scintillation monitor. Similar results were obtained in 3 experiments.

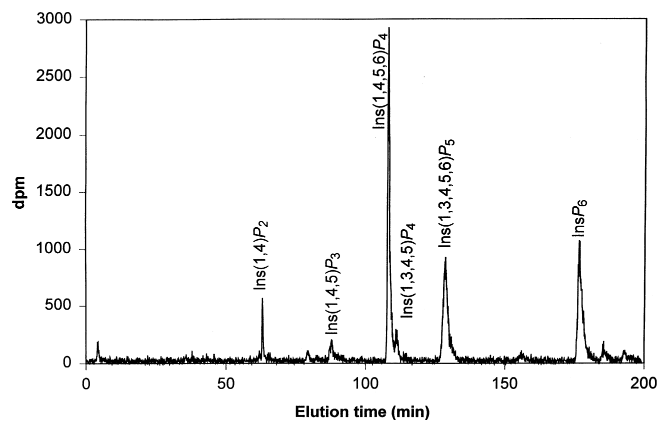


Figure 3 Products of ATP-dependent phosphorylation of Ins(1,4,5)P_3 by *S. pombe* soluble fraction

Soluble fraction (1.5 mg of protein) was incubated with [^{3}H]Ins(1,4,5)P_3 for 2 h and the products were separated by anion-exchange HPLC. Tentative identities, based on the elution positions of inositol polyphosphate standards, are indicated. Results of this type were obtained in 4 independent experiments.

When labelled stationary-phase yeasts were hyper-osmotically stressed, there was a rapid increase in the amount of [^{3}H]InsP_6 present. The InsP_6 concentration in these equilibrium-labelled cells approximately tripled within a few minutes, and an InsP_5 that co-chromatographed with Ins(1,3,4,5,6)P_5 became progressively more prominent after 30 min or more (Figure 2). These results identify InsP_6 as a normal constituent of *S. pombe*, whose concentration is environmentally regulated. The kinetics of this rapid increase in InsP_6 concentration in osmotically stressed cells would be consistent with InsP_6 synthesis from Ins(1,4,5)P_3 (see below). Little accumulation of labelled intermediates occurred during this synthesis. The most intense Ins(1,3,4,5,6)P_5 labelling occurred only after achievement of peak InsP_6 labelling, so this [^{3}H]Ins(1,3,4,5,6)P_5 may well have been a metabolite rather than a precursor of InsP_6. A more detailed analysis of this novel yeast response to osmotic stress will be reported elsewhere.

S. pombe extracts make InsP_6 from Ins(1,4,5)P_3

The coexistence of major peaks coincident with Ins(1,4,5)P_3 and InsP_6 could indicate that these compounds are inter-related in *S. pombe*. We therefore tested whether *S. pombe* extracts would metabolize Ins(1,4,5)P_3 to other inositol polyphosphates. When an *S. pombe* soluble fraction was incubated with a low concentration of [^{3}H]Ins(1,4,5)P_3 without added ATP, Ins(1,4,5)P_3 was slowly dephosphorylated, mainly to Ins(1,4)P_2 and Ins(4,5)P_2 (in varying proportions). Under first-order conditions, the half-

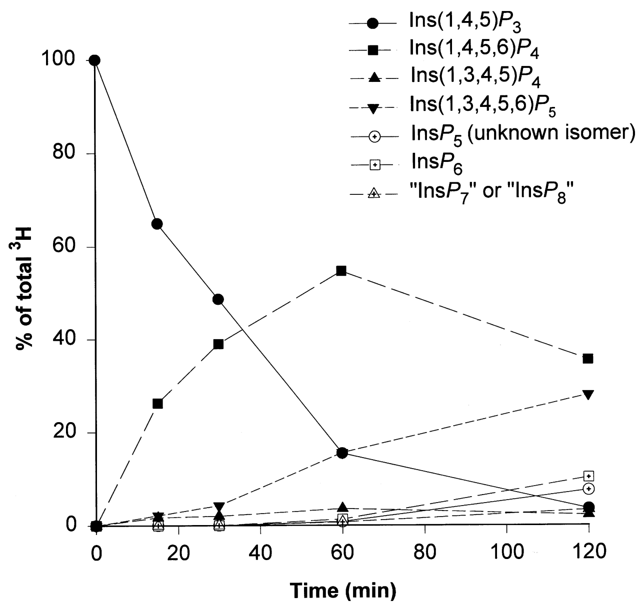


Figure 4 The time-course of inositol polyphosphate interconversions by *S. pombe* soluble fraction incubated with [^{3}H]Ins(1,4,5)P_3 and unlabelled ATP

The products were separated by HPLC and the identities indicated correspond to those assigned in the text and to peaks corresponding in elution positions to standard inositol polyphosphates. For simplicity, some minor products [Ins(1,4)P_2, Ins(4,5)P_2 and a third InsP_4], each of which constituted 3% or less of the products at all times, have not been plotted.

time ($t_{\frac{1}{2}}$) for Ins(1,4,5)P_3 dephosphorylation was $\approx$ 5 h (results not shown).

In ATP-supplemented incubations, [^{3}H]Ins(1,4,5)P_3 was quickly converted into more highly phosphorylated inositol derivatives, with a $t_{\frac{1}{2}}$ for Ins(1,4,5)P_3 consumption of $\approx$ 30 min. Ins(1,4,5)P_3 was efficiently converted to two InsP_4 isomers, an InsP_5 and InsP_6 (Figure 3). The time-courses of the accumulation of these compounds suggested a simple stepwise phosphorylation of Ins(1,4,5)P_3 to InsP_6 via InsP_4 and InsP_5 (Figure 4). Small amounts of labelled compounds with charges higher than InsP_6, possibly inositol polyphosphate pyrophosphates [23], were also formed. The major InsP_4 product eluted as Ins(1,4,5,6)P_4, and the minor InsP_4 as Ins(1,3,4,5)P_4. Ins(1,4,5,6)P_4, which was the major initial kinase product (see below), was not appreciably dephosphorylated by a yeast soluble fraction even when ATP was omitted. Under conditions in which Ins(1,4,5)P_3 was almost all phosphorylated, there was no detectable phosphorylation of [^{3}H]Ins(1,4)P_2.

A washed total particulate fraction metabolized Ins(1,4,5)P_3 only slowly: Ins(1,4,5)P_3 dephosphorylation was more rapid than in the soluble fraction, but there was little Ins(1,4,5)P_3 kinase activity (results not shown). Subsequent experiments focused on the soluble fraction.

Isolation of an *S. pombe* Ins(1,4,5)P_3 kinase

To investigate the route of Ins(1,4,5)P_3 metabolism further, we purified an Ins(1,4,5)P_3-phosphorylating kinase from the cytosol fraction by a multistep FPLC procedure involving chromatography on Mono S, a Zn^{2+} affinity column, a heparin affinity column and gel filtration through HR75–Sepharose (for details, see the Materials and methods section). The resulting kinase preparation, which was purified $\approx$ 10000-fold, eluted from the gel-filtration column with an apparent molecular mass of $\approx$ 40 kDa. SDS/PAGE analysis of the fractions of highest specific activity predominantly showed one polypeptide band of $\approx$ 41 kDa. The active kinase therefore seems likely to be a monomeric protein of $\approx$ 41 kDa. Attempts to obtain a partial amino acid sequence from the $\approx$ 41 kDa SDS/PAGE polypeptide band, as a prelude to cloning the Ins(1,4,5)P_3 6-kinase, have so far been unsuccessful. One partial amino acid sequence identified MRF1, a yeast mitochondrial peptide chain release factor ($\approx$ 40 kDa) that was purified by a very similar method [37], as a contaminant. A second, novel, N-terminal partial sequence failed to lead to successful cloning of the kinase.

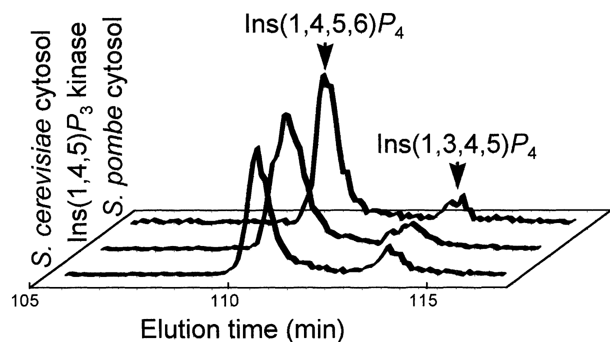


Figure 5 InsP_4 products made during brief Ins(1,4,5)P_3 kinase incubations

Three HPLC traces compare the InsP_4 region of chromatograms of the early products of the [^{3}H]Ins(1,4,5)P_3 kinase activities of *S. pombe* soluble fraction, the substantially purified *S. pombe* kinase and *S. cerevisiae* soluble fraction.

The purified kinase phosphorylates Ins(1,4,5)P_3 on the 6- or the 3-position

Whether our purest kinase preparation or a soluble fraction from either *S. pombe* or *S. cerevisiae* was used, two InsP_4 products were always formed in incubations containing [^{3}H]Ins(1,4,5)P_3 and unlabelled ATP (Figure 5). In our standard HPLC gradient (pH 4.4; see the Materials and methods section), the major [^{3}H]InsP_4 product (75–85% of the [^{3}H]InsP_4 products) eluted with Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4. This was earlier than the elution position of either Ins(1,3,4,5)P_4, the usual product of mammalian Ins(1,4,5)P_3 kinases, or Ins(1,3,4,6)P_4, another InsP_4 isomer commonly found in eukaryotic cells (Figure 5). The smaller, later eluting, peak co-eluted with authentic Ins(1,3,4,5)P_4. In an HPLC gradient at pH 3.8 in which Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4 eluted in the reverse order, the two InsP_4s formed by the kinase action reversed their elution order and again co-eluted with Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 (see below).

In principle, addition of a single phosphate to Ins(1,4,5)P_3 could yield Ins(1,2,4,5)P_4, Ins(1,3,4,5)P_4 or Ins(1,4,5,6)P_4. To confirm that the initial kinase products were Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4, we hydrolysed a mixed [^{3}H]InsP_4 fraction made up of the purified kinase with ammonia and analysed the liberated Ins$P$s. Ins1/3P, Ins4/6P and Ins5P species were formed, but no Ins2P was detected (results not shown), so the kinase can have made no Ins(1,2,4,5)P_4. Periodate oxidation, reduction and dephosphorylation converted the major [^{3}H]InsP_4 to [^{3}H]iditol, confirming it was Ins(1,4,5,6)P_4 (results not shown). The identity of the minor InsP_4 product was thereby confirmed as Ins-(1,3,4,5)P_4.

Since the relative proportions of Ins(1,4,5,6)P_4 and Ins-(1,3,4,5)P_4 produced were approximately constant at $\approx$ 5:1 whichever yeast cytosol was used and regardless of the purity of

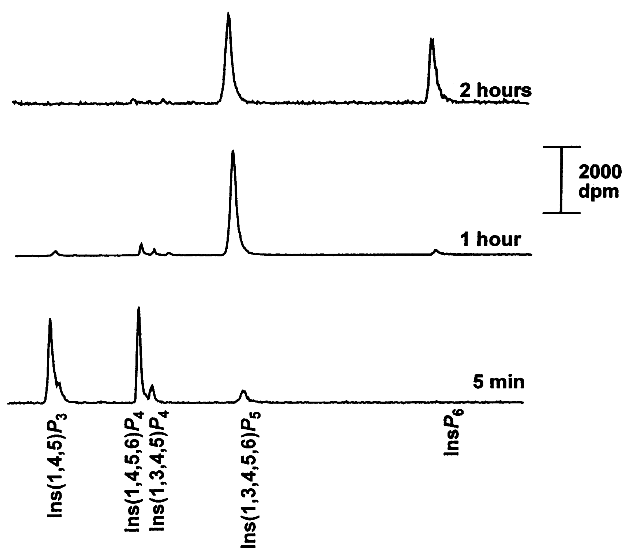


Figure 6 Products of [^{3}H]Ins(1,4,5)P_3 phosphorylation in prolonged incubations with the purified kinase preparation

Incubations were run under standard conditions for the periods indicated.

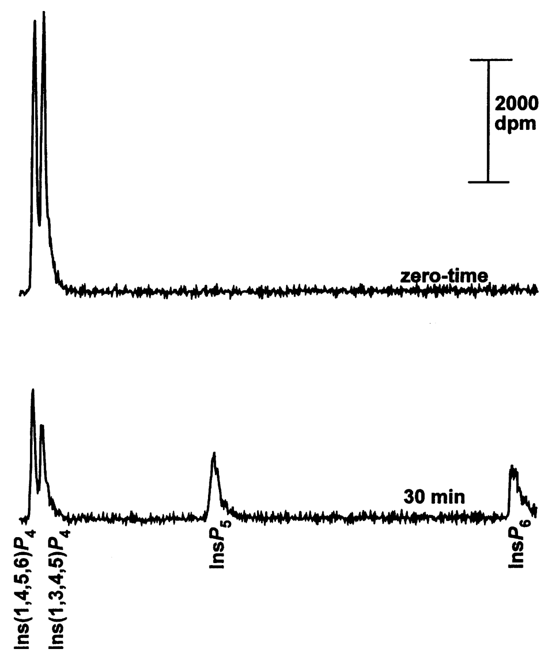


Figure 7 Conversion of both [^{3}H]Ins(1,4,5,6)P_4 and [^{3}H]Ins(1,3,4,5)P_4 into [^{3}H]InsP_5 and [^{3}H]InsP_6 by the purified kinase

The upper trace shows the mixture of Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 that was incubated with the purified kinase preparation, and the lower trace shows the products of this incubation.

the kinase, we conclude that each yeast contains a kinase that, in parallel, phosphorylates Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4 and to Ins(1,3,4,5)P_4.

Specificity and kinetic characteristics

Assays of the conversion of [^{3}H]Ins(1,4,5)P_3 to a mixed [^{3}H]InsP_4 fraction (isolated by simple Dowex chromatography) were used for kinetic analysis of the purified *S. pombe* kinase. It had a K_m for Ins(1,4,5)P_3 of $\approx 0.55\ \mu$M and for Mg^{2+}-ATP of $\approx 70\ \mu$M (results not shown). Phosphorylation was supported by several nucleoside triphosphates (ATP > GTP > > ITP, but not UTP), and was most rapid at pH 7.5–8.0. Increasing ionic strength inhibited activity, with $\approx 50\%$ inhibition at 0.2 M KCl.

Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 are further phosphorylated to an InsP_5 and InsP_6

During longer incubations of [^{3}H]Ins(1,4,5)P_3 with *S. pombe* cytosol, more highly phosphorylated ^{3}H-labelled products corresponding to an InsP_5 and to InsP_6 accumulated (Figure 3). This pattern of Ins(1,4,5)P_3 metabolism to multiple inositol polyphosphates, including InsP_6, was sustained through all stages of the kinase purification. In prolonged incubations with the most purified kinase preparations, Ins(1,4,5)P_3 was almost quantitatively converted to InsP_5 and InsP_6 (Figure 6). A comparison of the products of Ins(1,4,5)P_3 phosphorylation by the initial soluble fraction and the purified kinase revealed only minor differences: the purified kinase preparation did not dephosphorylate Ins(1,4,5)P_3 and did not make either a minor peak that eluted between the major InsP_5 and InsP_6 (a second, minor, InsP_5 product?) or the very polar metabolites that eluted after InsP_6.

The InsP_5 made by the kinase preparation was compared with Ins(1,3,4,5,6)P_5, the major InsP_5 made from Ins(1,3,4)P_3 via Ins(1,3,4,6)P_4 by a rat liver homogenate [32]. The two InsP_5 samples co-chromatographed, indicating that the *S. pombe* kinase activity makes Ins(1,3,4,5,6)P_5.

Either or both of Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4, produced by the *S. pombe* kinase, might be phosphorylated to Ins(1,3,4,5,6)P_5. Neither of these InsP_4s accumulated over prolonged periods, so it seemed likely that both were further phosphorylated. Each was therefore tested individually, and both were converted to Ins(1,3,4,5,6)P_5 and InsP_6: neither yielded any InsP_5 isomer other than Ins(1,3,4,5,6)P_5. When the purified kinase was offered a mixture of [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,4,5,6)P_4 (under first-order conditions), 6-phosphorylation of Ins(1,3,4,5)-P_4 was slightly faster than 3-phosphorylation of Ins(1,4,5,6)P_4 (Figure 7). When unlabelled Ins(1,3,4,5)P_4 or unlabelled Ins(1,4,5,6)P_4 (5 μM) was included in assays in which [^{3}H]Ins(1,4,5)P_3 was being phosphorylated under first-order conditions, each about halved the [^{3}H]InsP_4 yield, suggesting that their K_m values as substrates are $\approx 5\ \mu$M.

During conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5,6)P_5, the kinase is supplied with several times more Ins(1,4,5,6)P_4 than Ins(1,3,4,5)P_4, but phosphorylates the Ins(1,3,4,5)P_4 slightly more efficiently. We conclude that InsP_5 synthesis from Ins(1,4,5)P_3 proceeds mainly via Ins(1,4,5,6)P_4, but also via Ins(1,3,4,5)P_4.

Do other InsP_3 isomers inhibit Ins(1,4,5)P_3 phosphorylation?

We tested the ability of each of the possible regio-stereoisomers of InsP_3 to influence conversion of Ins(1,4,5)P_3 both to InsP_4 (using conditions in which $\leqslant 10\%$ of the initial InsP_4 products were further phosphorylated to InsP_5 and/or InsP_6) and onwards to InsP_5 and InsP_6.

At up to 100 μM, most of the inositol trisphosphates [Ins(1,2,3)P_3, Ins(1,2,4)P_3, Ins(1,2,5)P_3, Ins(1,2,6)P_3, Ins(1,5,6)P_3, Ins(2,4,5)P_3, Ins(2,4,6)P_3 and Ins(4,5,6)P_3] had no effect on the Ins(1,4,5)P_3 kinase reaction (results not shown). Ins(1,3,4)P_3 or Ins(1,3,5)P_3 (both 100 μM), which have three of the phosphate groups of Ins(1,3,4,5)P_4, approximately halved phosphorylation of [^{3}H]Ins(1,4,5)P_3 (≈ 1 nM; i.e. first-order reaction conditions). When the purified kinase was offered [^{3}H]Ins(1,3,4)P_3 as a

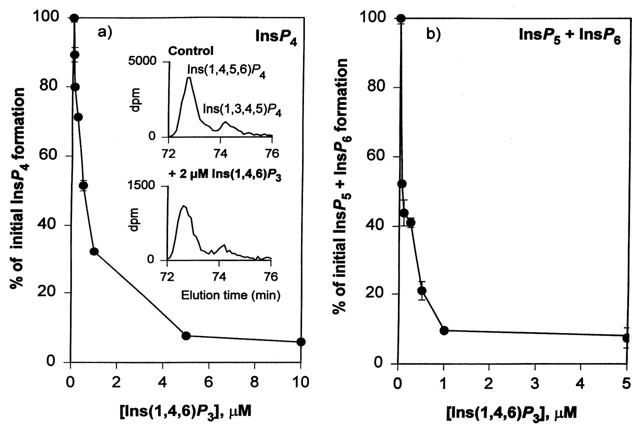


Figure 8 Inhibition of the purified Ins(1,4,5)P_3 kinase by Ins(1,4,6)P_3

The purified kinase was incubated with [^{3}H]Ins(1,4,5)P_3, ATP and various concentrations of Ins(1,4,6)P_3. There was progressive inhibition of Ins(1,4,5)P_3 conversion to InsP_4s (**a**) and InsP_5 + InsP_6 (**b**). The insets to **a** (note their different scales) show that Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 were produced at a ratio of $\approx$ 4:1 both in control assays and in assays with 2 mM Ins(1,4,6)P_3 ($\approx$ 70% inhibition).

potential substrate, it was not phosphorylated detectably (results not shown).

In sharp contrast, Ins(1,4,6)P_3 potently reduced the conversion of [^{3}H]Ins(1,4,5)P_3 to [^{3}H]InsP_4 under these conditions, with an IC_{50} of ≈ 0.7 μM (Figure 8a). Ins(1,4,6)P_3 also inhibited the further conversion of the initial InsP_4 products to InsP_5 plus InsP_6 (assayed as a combined fraction from Dowex mini-columns) with an even greater potency (IC_{50} 0.05–0.1 μM; Figure 8b). Under the first-order reaction conditions of these experiments, this greater inhibition of InsP_5 and InsP_6 synthesis was anticipated, since Ins(1,4,6)P_3 both limits the supply of [^{3}H]InsP_4 from Ins(1,4,5)P_3 and directly inhibits InsP_4 and InsP_5 phosphorylation.

The selectiveness of this inhibitory effect of Ins(1,4,6)P_3 only, of the many InsP_3 isomers tested, on all steps in conversion of Ins(1,4,5)P_3 into InsP_6, gives further support to the idea that one enzyme active site catalyses the formation of all of the products of Ins(1,4,5)P_3 phosphorylation.

Ins(1,4,6)P_3 is phosphorylated in the 5-position

Ins(1,4,6)P_3 might reduce [^{3}H]Ins(1,4,5)P_3 phosphorylation either by inhibiting the kinase or by being a competing high-affinity substrate. Since the kinase phosphorylates at least four different inositol polyphosphates on various positions (6-, 3- and 2-hydroxyls), it seemed likely that Ins(1,4,6)P_3 was another substrate. Before testing for Ins(1,4,6)P_3 phosphorylation, we compared molecular graphics representations of Ins(1,4,5)P_3, Ins-(1,3,4,5)P_4, Ins(1,4,5,6)P_4 and Ins(1,4,6)P_3, in the hope of predicting the point of attack. This comparison (for details, see the Discussion) suggested that the local environment of the 5-

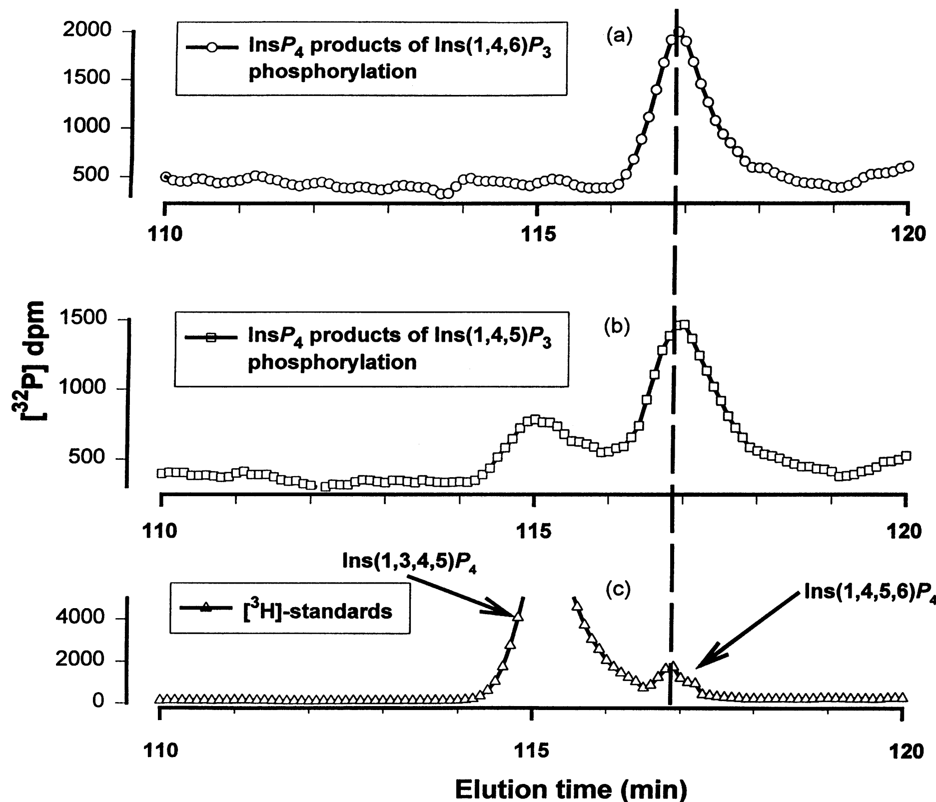


Figure 9 Products of the phosphorylation of Ins(1,4,5)P_3 and Ins(1,4,6)P_3 by [γ-^{32}P]ATP

Unlabelled (**a**) 5 μM Ins(1,4,6)P_3 or (**b**) 5 μM Ins(1,4,5)P_3 was incubated with 0.5 μM [γ-^{32}P]ATP and the inositol polyphosphate kinase for 2 h. Products were separated by HPLC, using a system in which Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 elute before Ins(1,4,5,6)P_4 (see the Materials and methods section). (**c**) The elution positions of [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,4,5,6)P_4. Incubations without added InsP_3 showed no [^{32}P]InsP_4 peaks.

hydroxyl of Ins(1,4,6)P_3 is like that of the 6-hydroxyl of Ins-(1,4,5)P_3, which the kinase phosphorylates fastest. We therefore predicted that our kinase might act as an Ins(1,4,6)P_3 5-kinase.

Labelled Ins(1,4,6)P_3 was not available as a substrate, so we compared the kinase-catalysed phosphorylation of unlabelled Ins(1,4,5)P_3 and Ins(1,4,6)P_3 with [γ-^{32}P]ATP. As expected, Ins(1,4,5)P_3 yielded two ^{32}P-labelled products corresponding to Ins(1,3,4,5)P_4 (minor) and Ins(1,4,5,6)P_4 (major) (Figure 9b). Because of the use of a different HPLC gradient (see the Materials and methods section), the order of elution of Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4 here is reversed compared with earlier Figures. Phosphorylation of Ins(1,4,6)P_3 gave a similar [^{32}P]InsP_4 yield, but only of a single [^{32}P]InsP_4 that co-chromatographed with Ins(1,4,5,6)P_4 and which was separated readily from Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 (Figure 9a). As predicted by the modelling studies, therefore, the *S. pombe* kinase 5-phosphorylated Ins(1,4,6)P_3.

DISCUSSION

The pathway to InsP_6 in *S. pombe* and the enzyme(s) involved

The above results show that *S. pombe* converts Ins(1,4,5)P_3 to InsP_6 by the pathway shown in Scheme 1. The major route is via Ins(1,4,5,6)P_4 (reactions 1, 3 and 5). Since Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 were made at a constant ratio throughout our kinase purification and in an *S. cerevisiae* extract, a single kinase almost certainly catalyses the Ins(1,4,5)P_3 6-kinase and 3-kinase reactions (reactions 1 and 2). This is a situation akin to that of the mammalian Ins(1,3,4)P_3 kinase that makes Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 in a $\approx 6:1$ ratio [38–40], and a related *Arabidopsis* Ins(1,3,4)P_3 kinase that makes Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 in a 1:5 ratio [41].

Does our single soluble *S. pombe* kinase also catalyse the other steps in the synthesis of InsP_6? This question will only be finally answered when our kinase is cloned, but all of our results strongly support the idea that it does. If it does not, then *S. pombe* (and probably also *S. cerevisiae*) must express, at the same relative concentrations, two Ins(1,4,5)P_3 3- and 6-kinases, two InsP_4 kinases and an Ins(1,3,4,5,6)P_5 kinase, all of which must all co-purify, and must be equally potently and specifically inhibited by Ins(1,4,6)P_3 and not by other inositol trisphosphates. This seems very unlikely.

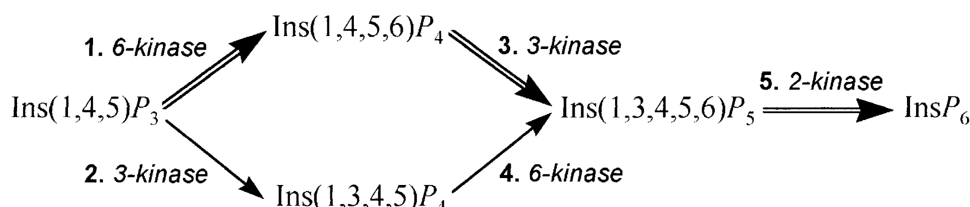
One recently contentious issue that these results resolve is whether yeasts can convert Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 [34,42]. It has previously been shown that *S. cerevisiae* contains an Ins(1,4,5)P_3 6-kinase [33], but it was also suggested that this yeast can make Ins(1,3,4,5)P_4 from Ins(1,4,5)P_3. However, the complete *S. cerevisiae* genome lacks any gene that would encode an Ins-(1,4,5)P_3 3-kinase of the type found as multiple isoforms in mammalian cells [42], and this fact has been used to argue that 3-phosphorylation of Ins(1,4,5)P_3 should not occur in yeasts. The results reported here reconcile this apparent contradiction by showing that *S. pombe*, and probably also *S. cerevisiae*, contain dual-specificity Ins(1,4,5)P_3 6/3-kinases that produce Ins-(1,3,4,5)P_4 as their less-abundant InsP_4 product and are distinct from Ins(1,4,5)P_3 3-kinases described previously [43]. This conclusion illustrates the fallacy of arguing that the absence of a recognizable enzyme of a particular type from a completed genome can demonstrate an organism's inability to carry out the reaction catalysed by the enzyme in question.

The environments of phosphorylatable hydroxyl groups

Our yeast kinase phosphorylates Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4,5,6)P_5 and Ins(1,4,6)P_3 on one or more of four hydroxyls (2-, 3-, 5- and 6-), but recognizes neither Ins(1,4)P_2 nor InsP_3 isomers other than Ins(1,4,5)P_3 and Ins(1,4,6)P_3 (for details, see Results). All identified substrates have at least one equatorial pair of *para*-phosphate groups [Ins(1,3,4,5,6)P_5 has two pairs], and most inositol phosphates that did not affect its kinase activity lack this feature.

To define the necessary features of hydroxyls acceptable by this kinase as substrates, we compared energy-minimized views, from the likely direction of approach of the kinase, of its substrates (Figure 10). The 6-hydroxyl of Ins(1,4,5)P_3 is phosphorylated fastest. On the 5,6 margin of Ins(1,4,5)P_3, flanked by the 1- and 4-phosphates, the equatorial 6-hydroxyl is 'tucked in' between the equatorial 1- and 5-phosphate groups (Figure 10a). When Ins(1,4,5)P_3 is viewed from the opposite side, the 1- and 4-phosphate groups, in reversed positions, bracket its 2,3 margin (Figure 10b). The environment of the 3-hydroxyl, the less-favoured substrate hydroxyl in Ins(1,4,5)P_3, has some similarities with that of the 6-hydroxyl, but here the axial 2-hydroxyl occupies a position similar to that of the much larger equatorial 5-phosphate on the other edge of the ring (compare Figures 10a and 10b). Ins(1,3,4,5)P_4 (Figure 10c) and Ins(1,4,5,6)P_4 (Figure 10d) are phosphorylated on the 6- and 3-hydroxyls, respectively, which have very similar environments in Ins(1,4,5)P_3 and the InsP_4s (compare Figures 10c and 10d with 10a and 10b). Neither Ins(1,3,4)P_3 nor Ins(1,4)P_2 is a substrate and Ins(1,3,4)P_3 inhibits Ins(1,4,5)P_3 phosphorylation only weakly.

Thus it seems that substrate hydroxyls in Ins(1,4,5)P_3, Ins-(1,4,5,6)P_4 or Ins(1,3,4,5)P_4 are all equatorial, lie in the 2-carbon span between a pair of *para*-phosphate groups and have a second phosphate or an hydroxyl on the other carbon in that span. Of the phosphorylation reactions we studied, only the final phosphorylation of the axial 2-hydroxyl of Ins(1,3,4,5,6)P_5 does not fit this description. We predicted that Ins(1,4,6)P_3 would be 5-phosphorylated (see the Results) because the 5-hydroxyl's situation looked similar to that of the 6-hydroxyl of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 (compare Figure 10e with Figures 10a and 10c). The success of this prediction suggests that this simple description does at least partly define the features needed for an hydroxyl to be a good substrate of this kinase.



Scheme 1 The pathway of Ins(1,4,5)P_3 conversion to InsP_6 in *S. pombe*

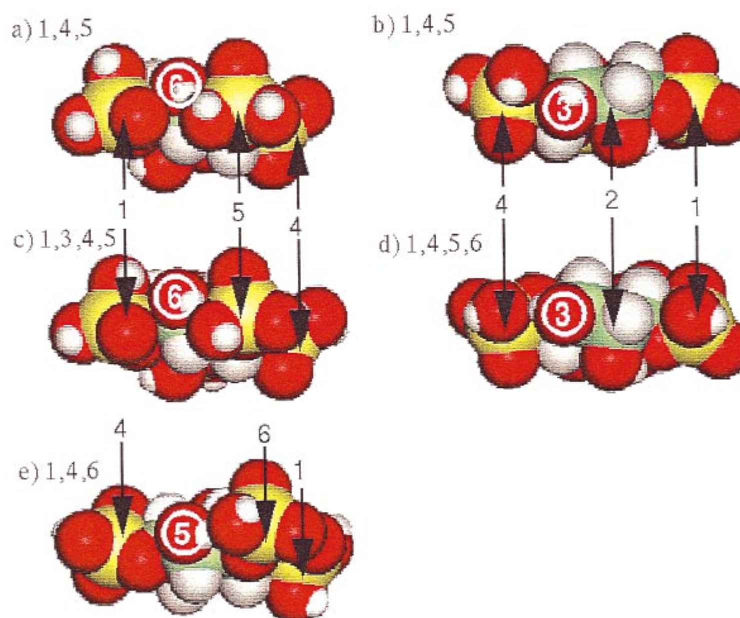


Figure 10 Edge-on views of energy-minimized molecular models of InsP_3 and InsP_4 isomers, viewed from the likely point of view of the catalytic site of the *S. pombe* inositol polyphosphate kinase

(**a**) Ins(1,4,5)P_3, looking at the 5,6 edge, with the 1-phosphate group to the left and the 4-phosphate to the right. (**b**) Ins(1,4,5)P_3, looking at the 2,3 edge, with the 4-phosphate group to the left and the 1-phosphate group to the right. (**c**) Ins(1,3,4,5)P_4, viewed as in (**a**). (**d**) Ins(1,4,5,6)P_4, viewed as in (**b**). (**e**) Ins(1,4,6)P_3, looking at the 5,6 edge, with the 4-phosphate group to the left and the 1-phosphate to the right.

A possible catalytic model

The key binding sites that orient the kinase at the 5,6 margin (Figures 10a and 10c) or 2,3 margin (Figures 10b and 10d) of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 or Ins(1,4,5,6)P_4 seem likely to be the 1-, 4- and 5-phosphate groups [for 6-phosphorylation of Ins-(1,4,5)P_3 and Ins(1,3,4,5)P_4; Figures 10a and 10c] or the 1- and 4-phosphates plus the 2-hydroxyl [for 3-phosphorylation of Ins-(1,4,5)P_3 or Ins(1,4,5,6)P_4; Figures 10b and 10d]. The $\approx 5:1$ preference for 6-phosphorylation of Ins(1,4,5)P_3 over its 3-phosphorylation presumably arises because the function required of the third grouping is filled better by a 5-phosphate than by a 2-hydroxyl. During phosphorylation of the InsP_4s, the previously added phosphate group will be 'around the back' of the substrate and probably has little effect on InsP_4 access to the catalytic site. However, Ins(1,4)P_2 is not a substrate, even for 3-phosphorylation, so groupings remote from those on the phosphorylated margin of the substrate must also play some role.

Consideration of the enzyme's handling of Ins(1,4,6)P_3 supports this 'three-grouping' view of substrate recognition. Ins-(1,4,5)P_3 is 5-phosphorylated, and 0.5 μM Ins(1,4,6)P_3 approximately halves the phosphorylation of [^{3}H]Ins(1,4,5)P_3 under first-order conditions, suggesting that it has an affinity for the substrate binding site similar to that of Ins(1,4,5)P_3 ($K_m \approx 0.55$ μM). When the appearances of the 5,6 margin of Ins(1,4,6)P_3 and the same margin of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 are compared, but with the Ins(1,4,6)P_3 image inverted relative to the other two images (compare Figure 10e with Figures 10a and 10c), the spatial context of the 5-hydroxyl of Ins(1,4,6)P_3 looks very like that of the 6-phosphate of either Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4. This led us to predict, correctly, that the 5-hydroxyl of Ins(1,4,6)P_3 should be a substrate for phosphorylation by this yeast kinase, even though this hydroxyl is already phosphorylated in all of the known natural substrates of this kinase [Ins(1,4,5)P_3, Ins-(1,3,4,5)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5].

InsP_6 in yeasts

We shall not know why most or all eukaryotic cells, including yeasts, make InsP_6 in substantial quantities until there is firm information on the biological role of this relatively abundant contributor to the cytosolic polyanion complement (for reviews, see [6,11–15,44–48]). Similarly, there are no clues why yeasts share with plants a synthetic route to InsP_6 from Ins(1,4,5)P_3, rather that using one of the pathways employed by mammalian cells. The signalling function of Ins(1,4,5)P_3 in stimulated animal cells is well understood, and in those cells the route from Ins(1,4,5)P_3 to InsP_6 is relatively long and much of the Ins(1,4,5)P_3 is dephosphorylated [directly or via Ins(1,3,4,5)P_4 and Ins-(1,3,4)P_3]. In contrast, we know neither why nor under what circumstances yeast makes Ins(1,4,5)P_3. The only real hint is that Ins(1,4,5)P_3 accumulates, presumably as a result of PtdIns(4,5)P_2 hydrolysis by phosphoinositidase C, when a nitrogen source is re-admitted to starved *S. cerevisiae* [22].

Whatever the control of Ins(1,4,5)P_3 synthesis in yeasts, however, the information presented here suggests that a major fate of Ins(1,4,5)P_3 in *S. pombe* is phosphorylation to InsP_6, and even to the pyrophosphates derived therefrom. Although there is evidence that Ins(1,4,5)P_3 can be slowly dephosphorylated in *S. pombe* ([34] and the results cited above, plus J. A. Stuart, P. J. Hughes and P. Ongusaha, unpublished work), the three recently recognized inositol polyphosphate 5-phosphatases of *S. cerevisiae* [29–31] all seem more likely to dephosphorylate PtdIns(4,5)P_2 [and possibly PtdIns(3,5)P_2?] than Ins(1,4,5)P_3. We have shown that hyper-osmotic stress accelerates InsP_6 synthesis in *S. pombe*,

most likely from Ins(1,4,5)P_3, and it will be interesting to learn whether the nutrient-regulated acceleration of Ins(1,4,5)P_3 formation in *S. cerevisiae* can fuel a similar increase in its InsP_6 complement. Moreover, the work of Lakin-Thomas on *Neurospora* [26] suggests that InsP_6 synthesis may be of some special importance to fungal cells. She showed that the cellular InsP_6 complement is maintained, or even rises, when the inositol supply to these cells is limited, even under conditions in which the PtdIns concentration falls substantially.

Yeast InsP_6 therefore seems to be made from Ins(1,4,5)P_3 by the most direct possible route, its concentration is protected in the face of inositol limitation and its synthesis can be driven by osmotic stress. All of these observations suggest that InsP_6 serves some important function(s) in yeasts, which may be similar to its function in other eukaryotic cells. Attempts to define InsP_6 function(s) have for a decade been hampered by the fact that the cellular InsP_3 concentration is usually influenced little by extracellular regulatory influences, and that InsP_6 turns over slowly. Our identification of a situation in which InsP_6 synthesis is acutely regulated should speed the definition of its function(s).

We are grateful to the Medical Research Council, the Lister Institute and the Royal Society for financial support, to Professor S.-K. Chung for isomeric inositol trisphosphates, to Dr. A. Pemberton for help with molecular graphics and to Dr. S. K. Dove for valuable discussions.

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Received 8 June 1998/29 July 1998; accepted 26 August 1998