Inositol trisphosphate metabolism in *Saccharomyces cerevisiae*: identification, purification and properties of inositol 1,4,5-trisphosphate 6-kinase

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Ins(1,4,5) P_3 metabolism was examined in *Saccharomyces cerevisiae* extracts. *S. cerevisiae* contains readily detectable Ins(1,4,5) P_3 kinase activity that is predominantly soluble, but phosphomonoesterase activity acting on Ins(1,4,5) P_3 was not detected in either soluble or particulate preparations from this organism. We have purified the kinase activity ~ 685-fold in a rapid four-step process, and obtained a stable preparation. The enzyme has an apparent native molecular mass of ~ 40 kDa, and displays Michaelis–Menten kinetics with respect to its two substrates, ATP and Ins(1,4,5) P_3 . The K_m for ATP was 2.1 mM,

and that for Ins(1,4,5) P_3 was 7.1 μ M. The enzyme appeared to be the first step in the conversion of Ins(1,4,5) P_3 into an Ins P_5 , and the partially purified preparation contained another activity that converted the Ins P_4 product into an Ins P_5 . The Ins P_4 product of the partially purified kinase was not metabolized by human erythrocyte ghosts and co-chromatographed with an Ins(3,4,5,6) P_4 [L-Ins(1,4,5,6) P_4] standard, identifying it as D-Ins(1,4,5,6) P_4 . The yeast enzyme is thus an Ins(1,4,5) P_3 6-kinase. This activity may be an important step in the production of inositol polyphosphates such as Ins P_5 and Ins P_6 in *S. cerevisiae*.

INTRODUCTION

Inositol phospholipids, especially PtdIns(4,5) P_2 , have critical roles in cellular control because they are substrates for receptor-regulated enzymes, such as phospholipases C and phosphatidylinositol 3-kinase, which generate established or putative intracellular signals (Nishizuka, 1984; Berridge and Irvine, 1989; Cantley et al., 1991; Downes and Carter, 1991; Stephens et al., 1993). However, relatively little progress has been made towards elucidating the functions of inositol-phospholipid-derived second messengers in complex cellular events such as the control of cell division. Such an objective may be facilitated by studying aspects of these signal-transduction pathways in genetically tractable organisms such as the budding yeast, *Saccharomyces cerevisiae*. For example, it is well established that adenylate cyclase in yeast is under environmental/nutritional control via the GTP-dependent protein products of the RAS1 and RAS2 genes and that a high concentration of cyclic AMP is required for entry into the cell cycle (Camonis et al., 1986; Broek et al., 1987; Munder and Kuntzel, 1988; Jones et al., 1990).

Several lines of evidence point to an important role for inositol phospholipids during execution of the cell cycle in *S. cerevisiae*. The well-known inositol phospholipids, PtdIns, PtdIns4 P and PtdIns(4,5) P_2 are present in yeast and appear to be essential for nutrient regulation of the cell cycle (Lester and Steiner, 1968; Talwalkar and Lester, 1973; Angus and Lester, 1975; McKenzie and Carman, 1983; Hawkins et al., 1993). Recently, *S. cerevisiae* has been shown to be a relatively abundant source of PtdIns3 P , one of the products of PtdIns 3-kinase (Auger et al., 1989; Hawkins et al., 1993). Moreover, PtdIns(4,5) P_2 -specific monoclonal antibodies, loaded intracellularly by electroporation, inhibited yeast cell proliferation (Uno et al., 1988).

Inositol phospholipids undergo enhanced metabolism when glucose-starved stationary-phase yeast cells are stimulated to re-enter the cell cycle by the addition of glucose (Steiner and Lester,

1972; Hawkins et al., 1993). However, this response is accompanied by the extracellular release of substantial quantities of deacylated inositol phospholipids and appears to result from the activation of one or more phospholipases A_2 and the presence of constitutively active lysophospholipase activity. An earlier report that Ins P_3 accumulates in glucose-stimulated yeast (Kaibuchi et al., 1986) used chromatographic techniques that would not distinguish between an Ins P_3 and deacylated PtdIns(4,5) P_2 . More recently, Ins(1,4,5) P_3 was shown to accumulate in nutrient-deprived *S. cerevisiae* in response to addition of an N source, but not in response to a C source such as glucose (Schomerus and Kuntzel, 1992). These results suggest that diverse pathways of inositol phospholipid metabolism may have roles in specific responses of yeast to nutrient stimuli, but the relationships between these functions and their counterparts in mammalian cells are not known at present.

In order to investigate the functions of Ins(1,4,5) P_3 in yeast and to devise strategies for genetic manipulations of its cellular concentration, we have looked for enzymes which recognize and metabolize this second messenger. We report the unexpected findings that *S. cerevisiae* contains Ins(1,4,5) P_3 6-kinase activity, but no detectable Ins(1,4,5) P_3 phosphomonoesterases or Ins(1,4,5) P_3 3-kinase. The Ins(1,4,5) P_3 6-kinase activity appears to be one component of a kinase cascade capable of synthesizing Ins P_5 from Ins(1,4,5) P_3 .

MATERIALS AND METHODS

Materials

[^{3} H]Ins(1,4,5) P_3 , myo-[^{14} C]inositol, [^{14} C]sorbitol, [^{32} P] P_i and Ins(1,4,5) P_3 were purchased from Amersham International, Amersham, Bucks., U.K. [^{3} H]Ins(1,3,4,5) P_4 , [^{3} H]Ins(1,4,5) P_3 were from NEN. Phenylmethanesulphonyl fluoride and alkaline phosphatase were from Boehringer Mannheim. Molecular-mass markers for gel-filtration chromatography, EGTA, NaF and

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Na_3VO_4 were purchased from Sigma. All other chemicals were of standard laboratory grade or better and were purchased from BDH, Poole, Dorset, U.K.

Preparation of *S. cerevisiae* cell extracts

Initial experiments explored the subcellular distribution of $\text{Ins}(1,4,5)\text{P}_3$ -metabolizing activities in yeast soluble and particulate fractions. Small-scale cultures of *S. cerevisiae* were grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose). The cell pellet was washed by resuspension in 10 mM KH_2PO_4 /150 mM NaCl, pH 7.4, three times. The washed cell pellet was resuspended in 2 vol. of ice-cold lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.1 mM Na_3VO_4 , 0.5 mM dithiothreitol and 1 mM phenylmethanesulphonyl fluoride, pH 7.4 at 4 °C). Homogenization was carried out by using an equal volume of chilled glass beads (0.4 mm diameter). The tubes containing the homogenization mixture were vortex-mixed for 2 min and then cooled on ice for 5 min. After three further cycles of cooling and vortex-mixing, the lysate was recovered from the glass beads by allowing the beads to settle for 10 min at 4 °C, and then removing the supernatant. The beads were then washed in an equal volume of homogenization buffer, and the resulting supernatant was combined with the lysate supernatant. Soluble fractions were prepared by centrifuging the resulting homogenate at 60000 rev./min for 30 min in a 70.1 Ti rotor in a Beckman L8-M ultracentrifuge. The particulate fraction also produced by this process was re-homogenized in the same buffer, centrifuged again and washed by this means a further twice.

For enzyme purification, 12-litre cultures of the protease-deficient *S. cerevisiae* strain ABYS106, grown in YPD medium, were harvested by centrifugation at 6000 rev./min in a Beckman J6B centrifuge for 10 min at 4 °C, and soluble fractions were prepared as above.

Purification of $\text{Ins}(1,4,5)\text{P}_3$ kinase

All procedures, except the h.p.l.c. anion-exchange and gel-filtration steps, were carried out at 4 °C. The supernatant obtained by centrifugation of yeast homogenates (1300 ml) was differentially precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ (percentage saturation values quoted are for solutions at 0 °C). Proteins which precipitated in the 30–60% -satd. $(\text{NH}_4)_2\text{SO}_4$ cut after 45 min on ice were collected by centrifugation, redissolved in buffer A (50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4 at 4 °C) and dialysed overnight against three successive changes of the same buffer.

The dialysed protein sample from the above procedure (150 ml) was applied to a TSK-DEAE column (150 ml bed volume) which was eluted at a flow rate of 3 ml/min with 150 ml of buffer A, followed by a gradient of 0–1 M NaCl (600 ml) in buffer A; 6 ml fractions were collected and assayed for $\text{Ins}(1,4,5)\text{P}_3$ kinase activity as described below. Fractions containing kinase activity were pooled and dialysed overnight against three successive changes each of 10 vol. of 10 mM KH_2PO_4 /1 mM dithiothreitol, pH 7.4 (buffer B).

A 30 ml portion of the dialysed protein sample was applied to a column containing 25 ml of hydroxyapatite (Bio-Gel HTP) that had been equilibrated with buffer B. The column was washed with 50 ml of buffer B and eluted with a linear gradient (240 ml) of 0–1 M potassium phosphate in buffer B at a flow rate of 2.6 ml/min; 4 ml fractions were collected and assayed for

$\text{Ins}(1,4,5)\text{P}_3$ kinase activity. The active fractions were pooled and dialysed overnight against three changes each of 10 vol. of buffer A.

The dialysed protein after hydroxyapatite chromatography was applied to a TSK-DEAE h.p.l.c. column which was then washed with several column volumes of buffer A. The column was eluted with a gradient composed of buffer A and buffer A plus 1 M NaCl according to the following protocol: 0 min, 0 M NaCl; 60 min, 0.4 M NaCl; 61 min, 1 M NaCl; 70 min, 1 M NaCl; 71 min, 0 M NaCl. The flow rate was 0.5 ml/min, and 1 min fractions were collected. Fractions containing $\text{Ins}(1,4,5)\text{P}_3$ kinase were pooled, dialysed extensively against buffer A and stored at 4 °C after addition of NaN_3 to 1 mM.

Determination of native molecular mass

A 0.1 ml portion of the h.p.l.c. DEAE-purified kinase was applied to a TSK 3000_{xl} h.p.l.c. gel-filtration column that had been equilibrated with buffer A containing 0.2 M NaCl at 20 °C. The column was eluted with this buffer at a flow rate of 0.2 ml/min, and 1 min fractions were collected. The gel-filtration column was calibrated with the following molecular-mass markers: thyroglobulin, 669 kDa; apoferritin, 443 kDa; β -amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; carbonic anhydrase, 29 kDa. Samples (0.025 ml) of each fraction were assayed for $\text{Ins}(1,4,5)\text{P}_3$ kinase activity.

Protein assays

Protein was determined by the method of Bradford (1976) with BSA as standard.

Assay of $\text{Ins}(1,4,5)\text{P}_3$ kinase activity

$\text{Ins}(1,4,5)\text{P}_3$ kinase was generally assayed under first-order conditions. Assays contained, in 0.1 ml, 5000–50000 d.p.m. of D- $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, 10 mM ATP, 30 mM NaCl, 70 mM KCl, 20 mM Hepes, pH 7.0, and either partially purified enzyme or crude fractions of yeast homogenates. Incubation times varied, but in kinetic analyses this was constrained to ensure that no greater than 20% of substrate was consumed. Assays were terminated by addition of 0.1 ml of ice-cold HClO_4 (6%, w/v). After 15 min on ice, 0.2 ml of a freshly prepared 1:1 (v/v) mixture of Freon/tri-n-octylamine was added, the samples were vortex-mixed thoroughly and then centrifuged to separate the phases. A 0.15 ml sample of the upper phase was analysed for radiolabelled inositol phosphates. Since all preparations used in these studies contained both $\text{Ins}(1,4,5)\text{P}_3$ kinase and InsP_4 kinase activities (see the Results section), the former was calculated as the accumulation of both InsP_4 and InsP_5 products.

Separation of inositol phosphates

Inositol phosphates were fractionated by anion-exchange chromatography on 0.5 ml columns of Bio-Rad AG1X8 (200–400 mesh; formate form) as described previously (Downes et al., 1986). Fractions (10 ml) containing radiolabelled inositol phosphates were mixed with an equal volume of Triton/xylene-based scintillant and counted for radioactivity by scintillation counting. For analysis by anion-exchange h.p.l.c., inositol phosphates were applied to a Partisphere SAX column which was eluted with a gradient made from buffers A (water) and B [1.25 M $(\text{NH}_4)_2\text{HPO}_4$ (adjusted to pH 3.8 with H_3PO_4 at 25 °C)] at a flow rate of 1 ml/min under the following conditions: 0 min, 0% B; 10 min, 0% B; 60 min, 20% B; 120 min, 100% B;

125 min, 100 % B; 130 min, 0 % B (gradient 1). An alternative (gradient 2) used in some experiments, was composed of buffers A (water) and B [1.5 M $(\text{NH}_4)_2\text{HPO}_4$ adjusted to pH 4.4 as above] at a flow rate of 1 ml/min under the following conditions: 0 min, 0 % B; 10 min, 0 % B; 15 min, 10 % B; 60 min, 50 % B; 65 min, 100 % B; 80 min, 100 % B; 81 min, 0 % B. Fractions (0.5 ml) were collected and counted for radioactivity by liquid-scintillation counting in either Optiphase Hi-safe 3 (Pharmacia) or Flowscint 4 (Packard).

Preparation of ^{32}P -labelled inositol phosphate standards

A mixture of InsP_4 s containing ^{32}P -labelled $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$ [$\text{L-Ins}(1,4,5,6)\text{P}_4$], and also $^{32}\text{P}[\text{InsP}_5]$, were prepared from turkey erythrocytes that had been labelled overnight with $^{32}\text{P}[\text{P}_i]$ (Stephens et al., 1988).

Structural analysis of inositol phosphates

Inositol phosphate products of enzyme reactions which had been purified by anion-exchange chromatography were first desalted. The samples were neutralized with triethylamine, diluted 10-fold with water, and applied to a column containing 0.2 ml of Bio-Rad AG1X8 (200–400 mesh; formate form). P_i (present in h.p.l.c. eluates) was eluted with 10 ml of 0.2 M ammonium formate/0.1 M formic acid. Inositol polyphosphates were then eluted by 2×2 ml of 2 M ammonium formate/0.1 M formic acid and desalted under vacuum as described by Carter and Downes (1993). Inositol phosphates were then characterized by anion-exchange h.p.l.c. at pH 4.4 (as described above). Inositol phosphates were also subjected to enzymic dephosphorylation by using human erythrocyte ghosts, which contain a well-characterized $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphomonoesterase activity. Haemoglobin-free human erythrocytes ghosts were prepared as described by Hawkins et al. (1984), and stored in 30 % glycerol/0.1 % mercaptoethanol at -70°C until required. Dephosphorylation reactions contained 0.08 ml of packed erythrocyte ghosts (approx. 0.4 mg of protein), and the radiolabelled inositol phosphate of interest in 0.2 ml of 15 mM Hepes/2 mM MgCl_2 /1 mM EGTA, pH 7.0. Incubations were at 37°C for the times indicated in the Figure legends. Reactions were processed for anion-exchange chromatographic procedures as described above.

Isolation of *S. cerevisiae* calmodulin

The *E. coli* plasmid pJG28, which expresses the CMD1 gene under the control of the *trc* promoter (gift from J. R. Geiser; see Geiser et al., 1991) was used to transform *E. coli* SURE. A 12.5-litre culture of the transformant was grown overnight at 37°C in LB medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin and 1 mM isopropyl β -D-thiogalactopyranoside. The crude bacterial lysate was heat-treated at 100°C for 5 min and denatured proteins were removed by centrifugation. The resulting supernatant was applied to a phenyl-Sepharose column in the presence of 0.1 mM Ca^{2+} . After washing with 10 bed vol. each of buffer 1 [0.05 M Tris/HCl (pH 7.5)/1 mM 2-mercaptoethanol] containing 5 mM CaCl_2 and buffer 1 containing 5 mM CaCl_2 and 0.5 M NaCl, calmodulin was eluted with buffer 1 containing 6 mM EGTA. Larger-molecular-mass contaminants were removed by gel filtration on a Superose 12 f.p.l.c. column equilibrated with 0.05 M Tris/HCl (pH 8.0), 1 mM EGTA, 0.1 M NaCl. Calmodulin-containing fractions were identified by immunoblotting against a crude anti-(yeast calmodulin) antiserum obtained from rabbits which had been immunized with a Protein A-calmodulin fusion protein.

Yeast calmodulin purified in this manner contained a single protein band of approx. 14 kDa on SDS/PAGE and activated mammalian Ca^{2+} /calmodulin-dependent protein kinase 6-fold in the presence of 0.1 mM Ca^{2+} (results not shown).

Isolation of calmodulin-binding proteins

Yeast calmodulin-binding proteins were prepared as described by Miyakawa et al. (1989), except that cells were homogenized in a Bead Beater (Biospec Products) as described by Stirling et al. (1992). Proteins retained on a DEAE column (150 ml bed volume) which were eluted in 0.22 M NaCl were collected, the CaCl_2 concentration was adjusted to 1 mM and the sample was applied to a bovine brain calmodulin affinity column (5 ml, containing 1 mg/ml calmodulin). The column was washed exhaustively with buffer A (20 mM Hepes/KOH, pH 7.0, 10 mM β -mercaptoethanol) containing 0.1 mM CaCl_2 , followed by buffer A plus 0.1 ml CaCl_2 and 0.2 M NaCl. Calmodulin-binding proteins were then eluted with buffer A containing 0.2 M NaCl plus 1 mM EGTA.

Western blotting

Analytical SDS/PAGE in 12 % acrylamide slab gels was performed as described previously (Laemmli, 1970). Proteins were transferred to polyvinylidene difluoride membranes (Millipore) as described by the manufacturer. Rainbow molecular-mass markers (Amersham) were used throughout.

RESULTS

Subcellular distribution of $\text{Ins}(1,4,5)\text{P}_3$ -metabolizing activities

$^3\text{H}[\text{Ins}(1,4,5)\text{P}_3]$ was incubated with crude cytosol and particulate fractions prepared from yeast homogenates, and the products of its metabolism were analysed initially by batch elution from small anion-exchange columns. The cytosol possessed readily detectable $\text{Ins}(1,4,5)\text{P}_3$ kinase activity that was dependent on the presence of MgATP. The elution characteristics of the products of the reaction indicated the production of both an InsP_4 and an InsP_5 (see below), and a time course of the reaction showed that there was a stoichiometric conversion of $^3\text{H}[\text{Ins}(1,4,5)\text{P}_3]$ into its more phosphorylated products (Figure 1), with the ratio of InsP_5 to InsP_4 increasing with time, suggesting a sequential phosphorylation pathway (see below). Very little $\text{Ins}(1,4,5)\text{P}_3$ -phosphorylating activity was present in the particulate fraction, and this was in any case sensitive to resuspension and washing in cell-lysis buffer, suggesting that it was due to contaminating soluble activity. A particularly surprising finding was the lack of detectable phosphomonoesterase activity in both particulate and soluble fractions when the assays were carried out in the presence or in the absence of Mg^{2+} or MgATP. Inositol bisphosphates, monophosphates or free inositol were not detected as products at any time during the incubations. This was confirmed by h.p.l.c. analysis of the products of $\text{Ins}(1,4,5)\text{P}_3$ metabolism by yeast cytosol, which gave three apparently homogeneous peaks, corresponding to residual $^3\text{H}[\text{Ins}(1,4,5)\text{P}_3]$, a single $^3\text{H}[\text{InsP}_4]$ peak and a single $^3\text{H}[\text{InsP}_5]$ peak (results not shown). These results suggest that the formation of inositol polyphosphates in these incubations results from the direct one-step phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$, followed by direct phosphorylation of the InsP_4 so produced. With the same assay conditions and protein content, $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase was readily detectable in rat brain cytosol.

Initial characterization of the $\text{Ins}(1,4,5)\text{P}_3$ -phosphorylating activity of yeast cytosol indicated that it was dependent on the presence of MgATP, but that increasing the Mg^{2+} concentration

Figure 1 Time course for the accumulation of products of $\text{Ins}(1,4,5)\text{P}_3$ metabolism in *S. cerevisiae* soluble fraction

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was incubated with a soluble fraction from *S. cerevisiae* prepared as described in the Materials and methods section. Assays were run for the times indicated, and the products were separated on Dowex AG1X8 (formate) resin. Data points are the means of duplicates in a single experiment repeated on three separate occasions, each time using a fresh yeast soluble fraction. Symbols: $\circ$, $\text{InsP}_3/\text{InsP}_6$; $\blacklozenge$, InsP_4 ; $\square$, InsP_3 ; $\blacktriangle$, InsP_2 ; $\blacksquare$, InsP .

Table 1 Summary of the purification of $\text{Ins}(1,4,5)\text{P}_3$ kinase

Purification step	Activity (arbitrary units)	Yield (%)	Sp. activity (units/mg)	Purification (fold)
Soluble fraction	215 000	100	5.4	1
30–60% satd. $(\text{NH}_4)_2\text{SO}_4$ cut	192 000	89	15.7	2.9
TSK-DEAE	174 350	81	162	29.3
Hydroxyapatite	86 175	40	351	65
HPLC-DEAE	77 575	36	3 861	689
HPLC-DEAE	19 394			
HPLC-gel filtration	17 455	32	19 505	3612

above that of ATP did not significantly increase enzyme activity. Kinase activity had a broad pH optimum centred around pH 7.0 and was essentially unaffected by Na^+ or K^+ salts up to 100 mM, either alone or in combination (results not shown). Ca^{2+} was slightly inhibitory at concentrations from 10 μM upwards (see below). Hence we chose approximately intracellular ionic conditions for the routine assay of $\text{Ins}(1,4,5)\text{P}_3$ kinase activity.

Purification of $\text{Ins}(1,4,5)\text{P}_3$ kinase activity

In order to define the properties, and in particular the products, of the yeast $\text{Ins}(1,4,5)\text{P}_3$ kinase activity more clearly, a substantial purification of the cytosolic activity was undertaken. Greater than 600-fold purification was achieved through differential precipitation with $(\text{NH}_4)_2\text{SO}_4$, TSK-DEAE anion-exchange chromatography, hydroxyapatite chromatography and an

Figure 2 Purification of *S. cerevisiae* $\text{Ins}(1,4,5)\text{P}_3$ kinase activity

(a) Chromatography on DEAE-Cellulose; (b) chromatography on hydroxyapatite; (c) HPLC on TSK-DEAE 5PW. Columns were eluted and InsP_3 kinase was assayed in column fractions as described in the Materials and methods section. Only those fractions containing significant activity are shown in the Figure for clarity. Basal activity was defined as less than 50 d.p.m. of InsP_4 . —, NaCl or phosphate gradient.

h.p.l.c. anion-exchange procedure (Figure 2 and Table 1). The purification was reproduced on five separate occasions up to and including the hydroxyapatite step and twice through all the steps indicated in Table 1.

Notable features of the purification are, first, the stability of the $\text{Ins}(1,4,5)\text{P}_3$ kinase activity. We obtained approx. 35% recovery of total kinase activity, with most of the losses occurring during chromatography through hydroxyapatite. However, significant instability of the activity was noted in pilot experiments before optimization of the homogenization and $(\text{NH}_4)_2\text{SO}_4$ precipitation procedures. The final preparation, however, was stable for several months under the storage conditions described. In addition, the preparation could be stored for long periods, with little loss of activity, as a precipitate in 60% satd. $(\text{NH}_4)_2\text{SO}_4$. It is therefore possible to accumulate the activity of several fermenter loads of yeast before undertaking large-scale purification. Second, the anion-exchange h.p.l.c. step gave a large improvement in specific activity of the preparation, even though a large-scale DEAE column was used earlier in the procedure. In order to achieve this, a very shallow salt gradient was used to elute the $\text{Ins}(1,4,5)\text{P}_3$ kinase activity.

Another important feature of the $\text{Ins}(1,4,5)\text{P}_3$ kinase preparation was the continued presence, throughout the purification, of a kinase that could phosphorylate the initially formed InsP_4 product to an InsP_5 . This is illustrated in Figure 3, which shows

Figure 3 Determination of the native molecular mass of $\text{Ins}(1,4,5)\text{P}_3$ kinase by h.p.i.c. gel filtration

A 0.1 ml portion of the h.p.i.c. DEAE-purified kinase was applied to a TSK 3000_{xl} h.p.i.c. gel-filtration column that had been equilibrated with buffer A containing 0.2 M NaCl at 20 °C. The column was developed with this buffer and InsP_3 kinase assays were carried out on column fractions as described in the Materials and methods section. Elution positions of molecular-mass standards are indicated in the inset. This experiment is representative of data for three separate preparations of the kinase. Symbols: ●, InsP_4 ; ○, InsP_5 ; △, $\text{InsP}_4 + \text{InsP}_5$. V_0 is the elution volume of individual marker proteins, and V_0 is the excluded volume, given by the elution volume of thyroglobulin.

the determination of the native molecular mass of the partially purified InsP_3 kinase preparation using gel-filtration chromatography. The presence of both $\text{Ins}(1,4,5)\text{P}_3$ and InsP_4 kinase activities in the purified preparation is indicated by the dip in the profile of InsP_4 product at the centre of the peak of activity. This dip is matched exactly by the profile of InsP_5 product in the same assays. This result shows that the $\text{Ins}(1,4,5)\text{P}_3$ and InsP_4 kinase activities have identical elution properties from this column, indicating that they share native molecular masses of approx. 40 kDa. Since, at this point in the purification, InsP_3 kinase activity was enriched, relative to crude cytosol, by greater than 3000-fold (Table 1), it is possible that the InsP_4 formed initially is a substrate for the same kinase that phosphorylates $\text{Ins}(1,4,5)\text{P}_3$. If a distinct InsP_4 kinase co-purifies with the $\text{Ins}(1,4,5)\text{P}_3$ kinase, it must be very similar in both native size and charge.

Identity of the product of $\text{Ins}(1,4,5)\text{P}_3$ kinase

The identity of the InsP_4 product of the $\text{Ins}(1,4,5)\text{P}_3$ kinase was investigated by determining its sensitivity to enzymic dephosphorylation and its chromatographic properties on anion-exchange h.p.i.c. The radiolabelled InsP_4 product of the yeast $\text{Ins}(1,4,5)\text{P}_3$ kinase was purified by anion-exchange h.p.i.c. and desalted. The product of the yeast $\text{Ins}(1,4,5)\text{P}_3$ kinase was completely resistant to hydrolysis by human erythrocyte ghosts for up to 90 min. Under the same incubation conditions, a $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ standard was hydrolysed > 90 %, yielding one major ^3H -labelled product, which had the expected mobility of $\text{Ins}(1,4,5)\text{P}_3$ on anion-exchange h.p.i.c. (results not shown). This suggests that the yeast InsP_4 is not $\text{Ins}(1,3,4,5)\text{P}_4$.

Figure 4 Co-chromatography of yeast InsP_4 with $\text{Ins}(1,4,5,6)\text{P}_4$

Approx. 1750 d.p.m. of the desalted sample of yeast $[\text{H}]\text{InsP}_4$ prepared by using partially purified InsP_3 kinase was mixed with ^{32}P -labelled turkey erythrocytes InsP_4 s [a mixture of $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(3,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$] and applied to a Partisphere SAX column, which was eluted at pH 4.4 as described in the Materials and methods section. Fractions (0.5 ml) were collected and counted for radioactivity by scintillation counting by using a dual-label $^3\text{H}/^{32}\text{P}$ d.p.m. programme: □, ^3H ; ◆, ^{32}P . The small peak appearing in the ^3H window which coincides with the peak of $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ is due to spill-over and not to the presence of significant levels of ^3H in these fractions.

The unknown InsP_4 was subjected to anion-exchange h.p.i.c. on a Partisphere SAX column at pH 4.4. Under these conditions $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(3,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$ standards from turkey erythrocytes are well resolved (Figure 4, and Stephens et al., 1991). The ^3H -labelled product of yeast $\text{Ins}(1,4,5)\text{P}_3$ kinase co-chromatographed with standard ^{32}P -labelled $\text{Ins}(3,4,5,6)\text{P}_4$. As the direct phosphorylation of D- $\text{Ins}(1,4,5)\text{P}_3$ could yield D- $\text{Ins}(1,3,4,5)\text{P}_4$, D- $\text{Ins}(1,4,5,6)\text{P}_4$ or D- $\text{Ins}(1,2,4,5)\text{P}_4$, and since the latter is eluted after $\text{Ins}(1,3,4,6)\text{P}_4$ under the chromatographic conditions employed (Stephens et al., 1991), this result establishes the identity of the yeast InsP_4 as D- $\text{Ins}(1,4,5,6)\text{P}_4$, the enantiomer of turkey erythrocyte D- $\text{Ins}(3,4,5,6)\text{P}_4$. The partially purified enzyme obtained from yeast cytosol is thus an $\text{Ins}(1,4,5)\text{P}_3$ 6-kinase.

Properties of $\text{Ins}(1,4,5)\text{P}_3$ 6-kinase

As noted above, the cytosolic $\text{Ins}(1,4,5)\text{P}_3$ kinase activity is dependent on MgATP for activity, has a broad pH optimum around pH 7 and is relatively insensitive to physiologically relevant univalent cations. Kinetic constants were determined under assay conditions in which not more than 20 % of $\text{Ins}(1,4,5)\text{P}_3$ substrate was consumed. The partially purified preparation (after the h.p.i.c. DEAE step) was essentially devoid of ATPase activity (results not shown), and consumption of ATP in these assays was negligible. $\text{Ins}(1,4,5)\text{P}_3$ 6-kinase displayed Michaelis–Menten kinetics with respect to both substrates. The results of six experiments using two separate enzyme preparations were analysed by using the Enzpack 3 program, and typical results are illustrated in Figure 5. The kinase has a relatively low K_m for $\text{Ins}(1,4,5)\text{P}_3$ of $7.1 \pm 0.87 \mu\text{M}$, which is consistent with the likely low levels of this compound in living cells. By contrast, the K_m for ATP was found to be $2.1 \pm 0.47 \text{ mM}$, suggesting that the enzyme might not be fully saturated with ATP under conditions that prevail in the cell. Alternatively, the enzyme's affinity for

Figure 5 Kinetic properties of Ins(1,4,5)P₃ 6-kinase

H.p.l.c. DEAE-purified enzyme was used to determine the K_m for both Ins(1,4,5)P₃ (a) and ATP (b) as described in the Materials and methods section. The results are means of duplicates in typical experiments, which were repeated six times for Ins(1,4,5)P₃ and three times for ATP, using two different preparations of enzyme. Data were fitted by the Enzpack 3 program.

Table 2 Effects of Ca²⁺/calmodulin on yeast Ins(1,4,5)P₃ 6-kinase

TSK-DEAE h.p.l.c.-purified Ins(1,4,5)P₃ kinase was assayed as described in the Materials and methods section, with no additions, 0.1 mM Ca²⁺ or 1 mM EGTA with or without 0.3 μM yeast calmodulin.

Additions	Ins(1,4,5)P ₃ kinase activity (% of control)
No additions	100
Ca ²⁺ /calmodulin	80 ± 5
EGTA/calmodulin	105 ± 1
Ca ²⁺	89 ± 5
EGTA	106 ± 3

ATP may be subject to regulatory mechanisms that are not reflected in these assays of the purified protein.

Most mammalian cells that have been analysed contain an Ins(1,4,5)P₃ 3-kinase that is stimulated by Ca²⁺/calmodulin (Li et al., 1989; Takazawa et al., 1989; Shears, 1989). Preliminary experiments suggested that the yeast Ins(1,4,5)P₃ 6-kinase was not affected by bovine brain calmodulin in either the presence or the absence of calcium. To confirm this result, and to ensure that insensitivity to calmodulin was not due to any species specificity, the results illustrated in Table 2 were obtained with *S. cerevisiae* calmodulin, purified as described in the Materials and methods section. The results show that Ca²⁺, at a concentration of 10 μM,

inhibited the kinase activity by about 25%, compared with controls having either no added Ca²⁺ or 1 mM EGTA and no added Ca²⁺. Calmodulin, whether in the presence or absence of Ca²⁺, had no detectable effect on the Ins(1,4,5)P₃ 6-kinase activity. We also investigated whether the purified preparation could be recognized by antibodies to rat brain Ins(1,4,5)P₃ 3-kinase, and a *S. cerevisiae* calmodulin-Protein A fusion protein, by Western blotting. Although partially purified rat brain InsP₃ kinase was recognized by the antibodies, no specific band was detected in the yeast enzyme preparation (results not shown). Similarly, the calmodulin-Protein A fusion protein detected a number of proteins purified from whole yeast as calmodulin-binding proteins (Stirling et al., 1992), but there was no specific band in the purified kinase preparation (results not shown). Finally, we isolated calmodulin-binding proteins from yeast cytosol by anion-exchange chromatography and Ca²⁺/calmodulin affinity chromatography and assayed for Ins(1,4,5)P₃ kinase activity. Activity was not detected in the fraction eluted from the affinity column with EGTA, in either the presence or the absence of Ca²⁺/calmodulin (results not shown). Taken together, these results suggest that the yeast Ins(1,4,5)P₃ 6-kinase is not regulated by Ca²⁺/calmodulin, and further that yeast cytosol does not appear to contain significant levels of a Ca²⁺/calmodulin-activated Ins(1,4,5)P₃ 3-kinase analogous to that in mammalian cells.

DISCUSSION

In mammalian cells the Ins(1,4,5)P₃ that is formed after receptor-stimulated hydrolysis of PtdIns(4,5)P₂ is metabolized by two principal routes. These involve Ins(1,4,5)P₃ 5-phosphomonoesterase and Ins(1,4,5)P₃ 3-kinase activities (Downes et al., 1982; Irvine et al., 1986; Shears, 1989). The relative activities of these two pathways for Ins(1,4,5)P₃ metabolism vary between cells and according to the stimulatory conditions. The kinase pathway is particularly important for several reasons. Firstly, it is a major feedback control point in the metabolism of the Ca²⁺-mobilizing second messenger, Ins(1,4,5)P₃, because its activity is stimulated in the presence of Ca²⁺/calmodulin (Li et al., 1989; Takazawa et al., 1989). Secondly, the initial product of this pathway, Ins(1,3,4,5)P₄, has been proposed as an auxiliary intracellular signal which may function in concert with its metabolic precursor to regulate intracellular free [Ca²⁺] (Irvine, 1990, 1991). Thirdly, the kinase pathway provides an indirect link to complex metabolic loops which control the synthesis of inositol polyphosphates such as InsP₆(s) and InsP₆ (Shears et al., 1987; Balla et al., 1987; Stephens et al., 1988; Menniti et al., 1993). These compounds appear to have a ubiquitous distribution among eukaryotic cells, but their intracellular roles are not yet understood. In order to improve our understanding of the importance of inositol phosphates in yeast, we have initiated attempts to isolate enzymes involved in metabolism of Ins(1,4,5)P₃. The results establish some unusual and unexpected features of Ins(1,4,5)P₃ metabolism in this organism.

In repeated experiments using cytosol and total particulate fractions from wild-type and protease-deficient *S. cerevisiae* strains, we failed to detect significant Ins(1,4,5)P₃ phosphomonoesterase activity. Indeed, in the absence of added MgATP, metabolism of Ins(1,4,5)P₃ by homogenates, soluble or particulate fractions, was barely detectable. Under the conditions and protein concentrations employed in these assays, the 5-phosphomonoesterase activity present in rat parotid-gland cytosol would hydrolyse 50% of Ins(1,4,5)P₃ under first-order conditions within a few minutes (Hawkins et al., 1986). It

is possible that phosphomonoesterase activities in *S. cerevisiae* are particularly labile and, in an attempt to limit possible effects of proteolysis, dephosphorylation and thiol-group oxidation, we used a protease-deficient strain (ABYS 106), and homogenized cells in the presence of phenylmethanesulphonyl fluoride, fluoride, orthovanadate and dithiothreitol. The presence of these reagents did not reveal detectable levels of $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activities.

In the presence of MgATP , cytosol fractions were shown to possess $\text{Ins}(1,4,5)\text{P}_3$ kinase activity. This enzyme was purified > 600-fold by differential $(\text{NH}_4)_2\text{SO}_4$ precipitation, TSK-DEAE, hydroxyapatite and h.p.l.c. DEAE chromatographic procedures. The partially purified kinase activity obeys Michaelis-Menten kinetics with respect to both $\text{Ins}(1,4,5)\text{P}_3$ and ATP substrates. The K_m for $\text{Ins}(1,4,5)\text{P}_3$ of approx. $7\ \mu\text{M}$ suggests this enzyme will efficiently phosphorylate $\text{Ins}(1,4,5)\text{P}_3$, even at the low levels that are likely to be present in cells [$\text{Ins}(1,4,5)\text{P}_3$ concentrations in mammalian cells are in the micromolar range].

The *S. cerevisiae* enzyme, however, differs fundamentally from the known $\text{Ins}(1,4,5)\text{P}_3$ kinases found in mammalian cells. Firstly, the purified yeast enzyme is insensitive to Ca^{2+} /calmodulin, and is not recognized by antibodies to rat brain $\text{Ins}(1,4,5)\text{P}_3$ kinase or a *S. cerevisiae* calmodulin-Protein A fusion protein on Western blots. A more important distinction, however, is that the product of $\text{Ins}(1,4,5)\text{P}_3$ phosphorylation is $\text{Ins}(1,4,5,6)\text{P}_4$ and not $\text{Ins}(1,3,4,5)\text{P}_4$. The occurrence of an $\text{Ins}(1,4,5)\text{P}_3$ 6-kinase in yeast, the recent observation of a similar activity in pea roots (Chattaway et al., 1992) and the apparent lack of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase in either of these organisms has potentially important implications for the evolution of inositol phosphate kinases and mechanisms of Ca^{2+} signalling.

The $\text{Ins}(1,4,5)\text{P}_3$ kinase co-purified with an InsP_4 kinase activity that phosphorylated the product of the former enzyme. The latter must therefore be an $\text{Ins}(1,4,5,6)\text{P}_4$ kinase, but we have not unequivocally identified the InsP_5 product of this activity. It is possible that the co-purifying $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,4,5,6)\text{P}_4$ kinase activities are both functions of a single enzyme that is capable of phosphorylating $\text{Ins}(1,4,5)\text{P}_3$ at two positions. This would seem unlikely, because the sequential formation of InsP_4 and then InsP_5 indicates that product release occurs efficiently when a single phospho-transfer reaction has taken place, and yet only one species of InsP_4 , the $\text{Ins}(1,4,5,6)\text{P}_4$ isomer, accumulated in our experiments. We conclude that yeast probably contain two rather similar enzymes catalysing the sequential phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to an InsP_5 . For this reason we have deferred a more complete exploration of the substrate specificity of the InsP_5 kinase until a homogeneous preparation is available.

As noted above, the functions of $\text{Ins}(1,4,5)\text{P}_3$ in *S. cerevisiae* have not yet been unequivocally established. The lack of a detectable phosphomonoesterase and the occurrence of concerted kinases that effect almost quantitative conversion of $\text{Ins}(1,4,5)\text{P}_3$ into an unidentified InsP_5 suggest the possibility that $\text{PtdIns}(4,5)\text{P}_2$ metabolism to $\text{Ins}(1,4,5)\text{P}_3$ in *S. cerevisiae* might function predominantly to provide an essential precursor for the synthesis of inositol polyphosphates. Similar studies in another genetically tractable eukaryote, the slime mould *Dictyostelium discoideum*, identified a complete pathway capable of synthesizing InsP_6 from *myo*-inositol (Stephens and Irvine, 1990). The starting point for this pathway was the direct phosphorylation of *myo*-inositol by a cytosolic 3-kinase. However, we could not detect any *myo*-inositol kinase activity in yeast cytosol (A. N. Carter and C. P. Downes, unpublished work). The synthesis of InsP_5 and InsP_6 in mammalian cells is probably accomplished indirectly after $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis to $\text{Ins}(1,4,5)\text{P}_3$ by a sequence of reactions involving $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,6)\text{P}_4$

as intermediates (Shears et al., 1987; Balla et al., 1987; Stephens et al., 1988). It seems therefore that the ubiquitous occurrence of inositol polyphosphates in diverse eukaryotic organisms may reflect the existence of multiple pathways and involve distinct critical precursors.

In the budding yeast it seems likely that one function of $\text{Ins}(1,4,5)\text{P}_3$ is to provide a relatively direct precursor of, at least, InsP_5 . Future studies are planned to purify the yeast $\text{Ins}(1,4,5)\text{P}_3$ 6-kinase to homogeneity and to clone its gene. This strategy should allow genetic manipulation of the expression of this enzyme and provide important clues to the function, not only of $\text{Ins}(1,4,5)\text{P}_3$, but also of its phosphorylation products in *S. cerevisiae*.

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