Metabolism of inositol 1,4,5-trisphosphate in *Candida albicans*: significance as a precursor of inositol polyphosphates and in signal transduction during the dimorphic transition from yeast cells to germ tubes

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The metabolism of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] was examined in yeast cells and germ tubes of *Candida albicans*. Methods have been developed for analysis of the two key metabolic enzymes, Ins(1,4,5)P₃ kinase and phosphatase. ATP-dependent Ins(1,4,5)P₃ kinase activity was detected predominantly in the soluble fraction of cell extracts and exhibited a *K_m* of approximately 9 µM. The apparent *K_m* of Ins(1,4,5)P₃ phosphatase for Ins(1,4,5)P₃ was approximately 480 µM. The slow rate of dephosphorylation of Ins(1,4,5)P₃ to inositol bisphosphate suggests a lower importance of the phosphatase within cells compared to the kinase. Since both yeast cells and germ tubes of *C. albicans* rapidly phosphorylated Ins(1,4,5)P₃ to inositol tetrakisphosphate and inositol penta/hexakisphosphate, it is suggested that Ins(1,4,5)P₃ has an important role as a precursor for production of these compounds. A sustained increase in cellular Ins(1,4,5)P₃ levels was observed during germ tube formation and, prior to the onset of germination between 1 and 2 h incubation, the Ins(1,4,5)P₃ content increased up to eightfold. Transient increases in the level of Ins(1,4,5)P₃ were also observed during yeast-like growth of *C. albicans*. The possible role and relative importance of Ins(1,4,5)P₃ as a precursor for inositol polyphosphates and in signal transduction involving Ca²⁺ release from internal stores is discussed.

Keywords: inositol 1,4,5-trisphosphate, inositol 1,4,5-trisphosphate kinase and phosphatase, yeast–hyphal dimorphism, signal transduction, *Candida albicans*

INTRODUCTION

The process of eukaryotic cell communication, as well as sensing of the external environment, is mediated by the conversion of extracellular stimuli or signals into events within the cells. One such signalling pathway is the receptor-activated stimulation of phospholipase C,

Abbreviations: [Ca²⁺]_i, free cytosolic Ca²⁺; CaM, calmodulin; DAG, diacylglycerol; InsP₂, inositol bisphosphate; InsP₃, inositol trisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; InsP₄, inositol tetrakisphosphate; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetrakisphosphate; InsP₅, inositol pentakisphosphate; InsP₆, inositol hexakisphosphate; PtdIns, phosphatidylinositol; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate. (The non-specific abbreviations are used in contexts where unequivocal identification of the inositol phosphates was not known.)

which triggers the hydrolysis of phosphatidylinositol (PtdIns) bisphosphate [PtdIns(4,5)P₂], resulting in the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] (Berridge, 1987, 1993). DAG activates protein kinase C while Ins(1,4,5)P₃ stimulates the release of intracellular Ca²⁺ from non-mitochondrial Ins(1,4,5)P₃-sensitive calcium stores (Berridge, 1993). In fungi, glucose regulation of phospholipid synthesis has been reported in *Saccharomyces cerevisiae* (Kaibuchi *et al.*, 1986) with PtdIns(4,5)P₂ being found to be essential for yeast growth (Uno *et al.*, 1988). The plasma membrane of *S. cerevisiae* can synthesize CDP-DAG and PtdIns, as well as phosphorylating the latter compound (Kinney & Carman, 1990; Flanagan & Thorner, 1992). A gene (*PLC1*) which encodes a putative

phosphoinositide-specific phospholipase C has been isolated from *S. cerevisiae* and is important for cell growth, providing further evidence for signal transduction systems mediated by phospholipid hydrolysis in this organism (Yoko-o *et al.*, 1993). Furthermore, Kato *et al.* (1989) have shown that cAMP-dependent phosphorylation activates PtdIns kinase and PtdIns-4-phosphate kinase, which may lead to the production of Ins(1,4,5)P₃ and DAG. Such observations confirm a link between inositol-phospholipid-mediated and cAMP-mediated signalling systems; the multiplicity of dynamic interactions between different signalling systems should not be overlooked (Nishizuka, 1984, 1992; Gadd, 1995). In addition, inositol phospholipids appear important for execution of the cell cycle in *S. cerevisiae* and undergo enhanced metabolism when starved cells re-enter the cell cycle on glucose addition (Hawkins *et al.*, 1993). Rather less work has been carried out with fungi other than *S. cerevisiae*, although data are now accumulating on the nature and identity of inositol phosphates in filamentous fungi (Hanson, 1991; Prior *et al.*, 1993) as well as their possible direct/indirect involvement in signal transduction pathways (Brunton & Gadd, 1991; Gadd, 1995). The existence of key components and intermediates of the phosphoinositide signalling system have been demonstrated in several filamentous fungi including *Fusarium graminearum* (Robson *et al.*, 1991a, b; Prior *et al.*, 1993) and *Neurospora crassa* (Hanson, 1991), while indirect evidence for inositol-phospholipid-linked signal transduction during filamentation/germ tube formation has been obtained for *Candida tropicalis* (Kamihara & Omi, 1989) and *Ophiostoma ulmi* (Brunton & Gadd, 1991).

Ca²⁺ is now accepted to be an important intracellular messenger in eukaryotic cells (Berridge & Irvine, 1989; O'Day, 1990). However, while a multiplicity of Ca²⁺ [and Ca²⁺-calmodulin (CaM)]-mediated effects on fungal growth, differentiation and reproduction have been documented (see Robson *et al.*, 1991a; Jackson & Heath, 1993; Gadd, 1995), detailed confirmation of the involvement of Ca²⁺ in signal transduction in fungi is not as advanced as that of mammalian systems, in part due to methodological difficulties in the measurement of cytosolic free Ca²⁺ ([Ca²⁺]_c) (Knight *et al.*, 1993) as well as a plethora of non-specific effects caused by reagents and methodologies used to probe Ca²⁺ 'metabolism' (Youatt, 1993). However, the cellular mechanisms underlying Ca²⁺ regulation and homeostasis are reasonably understood with fungi utilizing a complex system of sequestrative and homeostatic mechanisms which maintain potentially toxic [Ca²⁺]_c at a low level (see Gadd, 1995). Such mechanisms involve transport phenomena (influx and efflux) at plasma and organellar membranes, calcium-binding proteins, of which CaM appears to be the most important and ubiquitous in fungi (Hubbard *et al.*, 1982; Trinci *et al.*, 1990; Pitt & Kaile, 1989; Liu *et al.*, 1990; Anraku *et al.*, 1991), and organellar compartmentation. In fungi, the vacuole(s) appears to be an important intracellular store for Ca²⁺ (Eilam *et al.*, 1985; Ohsumi *et al.*, 1988; Miller

et al., 1990; Bertl & Slayman, 1990; Anraku *et al.*, 1991) and it is upon such an intracellular Ca²⁺ store that Ins(1,4,5)P₃ is presumed to act; Ins(1,4,5)P₃-mediated Ca²⁺ channels on the vacuolar membrane have been demonstrated in *N. crassa* (Cornelius & Nakashima, 1987; Cornelius *et al.*, 1989), *S. cerevisiae* (Belde *et al.*, 1993) and *Candida albicans* (Calvert & Sanders, 1995). However, there is little detailed information for other fungi while some evidence also exists for an involvement of other cellular compartments such as the fungal endoplasmic reticulum (Rudolph *et al.*, 1989; Goffeau *et al.*, 1990; Halachmi *et al.*, 1992; Okorokov, 1994) and mitochondria (Pitt & Barnes, 1993) in intracellular Ca²⁺ homeostasis.

In this work, we have examined Ins(1,4,5)P₃ metabolism in the yeast-mycelium transition of the pathogenic yeast *C. albicans*. Simple *in vitro* regulation of the dimorphic transition, i.e. the production of germ tubes by unicellular yeast cells, in *C. albicans* by pH and/or temperature control has facilitated the study of a variety of cell processes associated with dimorphism in this organism. These include Ca²⁺-CaM interactions (Sabie & Gadd, 1989), Ca²⁺ uptake (Holmes *et al.*, 1991), thigmotropism (Sherwood *et al.*, 1992), ionic currents and galvanotropism (Crombie *et al.*, 1990; Lever *et al.*, 1994; Gow, 1994), all phenomena where signal transduction pathways have been implicated (Gow, 1994, 1995). We have therefore examined changes in cellular Ins(1,4,5)P₃ during the transition from yeast cells to germ tubes and developed methods for extraction and characterization of Ins(1,4,5)P₃ kinase and phosphatase, two key enzymes of Ins(1,4,5)P₃ metabolism, in order to investigate the metabolism and possible involvement of an inositol phosphate signal transduction mechanism in the dimorphic transition of *C. albicans*.

METHODS

Organism and growth conditions. *Candida albicans* 3153A was obtained from N. A. R. Gow (University of Aberdeen, UK). Routine maintenance was carried out at 25 °C on MYGP agar consisting of (g l⁻¹): malt extract (Lab M), 3; yeast extract (Oxoid), 3; bacteriological peptone (BDH), 5; D-glucose, 10; agar (Lab M, no. 2), 15. For liquid cultures, the following defined medium was used, consisting of (g l⁻¹): D-glucose, 12.5; (NH₄)₂SO₄, 5; MgSO₄·7H₂O, 0.2; K₂HPO₄ (anhydrous), 2.5; NaCl, 5.0; D-biotin, 0.001. The pH of the medium was adjusted after autoclaving using either 0.1 M HCl or 0.1 M NaOH. Starter cultures were prepared by loop-inoculating 100 ml liquid medium (pH 6.0), which was incubated for 48 h at 25 °C on a rotary incubator (150 r.p.m.). For experiments, 100 ml liquid medium was inoculated from a late-stationary-phase starter culture to an OD₅₅₀ of approximately 0.8 (~8 × 10⁶ cells ml⁻¹), measured using a Pye Unicam SP600 series 2 spectrophotometer. Germ tube formation by yeast cells of *C. albicans* was induced using pH and temperature regulation as described by Buffo *et al.* (1984). Growth of yeast cells resulted under the following conditions: pH 4.5, 25 °C; pH 4.5, 37 °C; pH 6.7, 25 °C. To induce germ tube formation, the medium was adjusted to pH 6.7 and the cells were incubated at 37 °C. Cells were counted using a modified Fuchs-Rosenthal haemocytometer after suitable dilution with distilled water.

Ins(1,4,5)P₃ determination. Yeast cells and germ tubes grown as described above were harvested from the liquid medium at 15 min intervals for the first 2 h after inoculation and then every hour, up to and including 5 h, by rapidly filtering onto cellulose nitrate membrane filters (0.45 µm pore size; 47 mm diameter). The filter with cells was then directly immersed in 2 ml 5% (v/v_{aq}) ice-cold HClO₄. One millilitre of the resulting cell suspension was vortexed at 4 °C in a test tube containing 0.5 g chilled glass beads (0.5 mm diameter) for 10 min, resting on ice for 30 s at 30 s intervals. Cell homogenates were then centrifuged (5 min, 2000 g) using an Eppendorf 5415 micro-centrifuge. Supernatant (400 µl) was then added to 100 µl 10 mM EDTA (pH 7.0) and to this, 900 µl of a 1:1 (v/v) freon and tri-*n*-octylamine mixture was added for neutralization. Each cell extract was vortexed vigorously for 30 s prior to centrifuging (1 min, 2000 g). The resulting top layer (400 µl) was removed for subsequent Ins(1,4,5)P₃ analysis using an Ins(1,4,5)P₃ radioligand [³H]assay system (TRK 1000; Amersham). The assay is based on the competition between unlabelled Ins(1,4,5)P₃ and a fixed quantity of [³H]-labelled Ins(1,4,5)P₃ for binding sites on a binding protein. With fixed amounts of binding protein and radioactive ligand, the amount of the latter bound is inversely proportional to the concentration of non-radioactive ligand. Bound Ins(1,4,5)P₃ is separated from free ligand by centrifugation and, after discarding the supernatant, measurement of radioactivity in the tube enables the amount of unlabelled Ins(1,4,5)P₃ in the sample to be determined from a standard curve. The assay can measure Ins(1,4,5)P₃ in the range 0.19–25 pmol (0.08–10.5 ng) per tube.

Preparation of cell extracts for enzyme assays. Yeast cells and germ tubes were harvested by filtration, 1.5 and 5 h after inoculation, as described above. These times correspond to the times of early and maximum germ tube formation. Cells were washed from the filters using 3 ml ice-cold homogenization buffer [20 mM HEPES; 1 mM Na₃VO₄; 2 mM EDTA; 2 mM EGTA; pH 7.0 (adjusted using 0.1 M NaOH) at 4 °C] and disrupted using 1.5 g chilled glass beads as described above. The cell homogenate was removed and the beads were washed with a further 1 ml buffer. The combined cell homogenate was centrifuged at 4 °C (30 min, 100 000 g) using a Sorvall ultracentrifuge (OTD65B). The supernatant was removed for use in Ins(1,4,5)P₃ kinase assays and as a source of soluble Ins(1,4,5)P₃ phosphatase activity. The pellet was resuspended in the original volume of homogenization buffer, recentrifuged at 4 °C (30 min, 100 000 g) as described above, washed a further two times and used for Ins(1,4,5)P₃ phosphatase determinations. Preliminary work showed that approximately 50% of all phosphatase activity was found in the soluble fraction and approximately 50% in the particulate fraction. For kinase assays, most activity (approx. 90%) was associated with the soluble fraction.

Ins(1,4,5)P₃ kinase and phosphatase assays. For determinations of kinase activity, soluble fractions of cell extracts were incubated in a water-bath at 25 or 37 °C with: 0.01–0.05 µCi (0.37–1.85 kBq) D-[³H]Ins(1,4,5)P₃ (Amersham), 30 mM NaCl, 120 mM KCl, 25 mM HEPES, 20 mM MgSO₄·7H₂O and 10 mM ATP, pH 7.0 (adjusted using 0.1 M NaOH), in a final volume of 100 µl. To measure Ins(1,4,5)P₃ phosphatase activity, soluble or particulate fractions of cell homogenates were incubated as above, omitting the ATP and using 1 mM MgSO₄·7H₂O. After required incubation times, assays were terminated by the addition of 100 µl 6% (v/v_{aq}) HClO₄, vortexed and centrifuged (2 min, 15 000 g) using a Sorvall Microspin 24. The supernatant was removed and neutralized using 200 µl of a 1:1 (v/v) mixture of freon and tri-

n-octylamine, vortexed for 30 s and centrifuged (2 min, 15 000 g). The top layer was then transferred to small anion-exchange columns (230 × 6 mm) for separation. The columns (obtained from Burkard Scientific) were packed with Bio-Rad AG 1-X8 resin beads (200–400 mesh, formate form) and the [³H]inositol-phosphate-labelled water-soluble components were separated with ammonium formate/formic acid eluants, according to the procedure of Morris *et al.* (1988) and using radiolabelled standards. Free inositol was eluted with 10 ml water, inositol monophosphate with 10 ml 0.2 M ammonium formate/0.1 M formic acid, inositol bisphosphate (InsP₂) with 10 ml 0.5 M ammonium formate/0.1 M formic acid, inositol trisphosphate (InsP₃) with 10 ml 0.75 M ammonium formate/0.1 M formic acid, inositol tetrakisphosphate (InsP₄) with 10 ml 0.9 M ammonium formate/0.1 M formic acid and inositol penta/hexakisphosphate (InsP_{5/6}) with 10 ml 2.0 M ammonium formate/0.1 M formic acid. Fractions (5 ml) of each wash, excluding the 2.0 M ammonium formate/0.1 M formic acid were added to 10 ml Optiphase 'Hi-Safe' 3 (Pharmacia) scintillation fluid and measured for radioactivity by scintillation counting. The 2.0 M ammonium formate/0.1 M formic acid eluant was mixed with an equal volume of water prior to removing 5 ml aliquots for scintillation counting as described above. It should be noted that this method of separation does not distinguish between different isomers of the inositol phosphates in each fraction, hence the non-specific abbreviations in this context.

Determination of affinities of Ins(1,4,5)P₃ kinase and phosphatase for Ins(1,4,5)P₃. Reaction mixtures for kinase assays were as described previously but with the addition of 0.1 µM–0.1 mM Ins(1,4,5)P₃. To calculate affinities, yeast cells only were used (grown for 5 h at 25 °C, pH 6.7, as described previously). Other experiments revealed that there were no major differences in affinities with enzymes assayed in extracts from cells forming germ tubes (results not shown). All samples were incubated at 25 °C. The initial rate of InsP₄ production was determined and was found to be less than 10% conversion of substrate over 5 min. For specific activity determinations of Ins(1,4,5)P₃ kinase, the substrate concentration was increased to three times the K_m value. To determine if Ins(1,4,5)P₃ kinase activity was regulated by different conditions of pH and temperature, cell extracts were incubated as described above except that all incubations contained Ins(1,4,5)P₃ at concentrations approximately 0.1 of the K_m value, since in order to find out if the enzyme is regulated by growth conditions (pH, temperature), saturating substrate concentrations (required for determining specific activity), which may mask any effects, should be avoided. To obtain the K_m value of Ins(1,4,5)P₃ phosphatase, cell extracts were incubated as described previously in the presence of 0.1 mM–1.5 mM Ins(1,4,5)P₃. The rate of InsP₂ production was measured (less than 10% conversion of substrate over 1 h).

RESULTS

Ins(1,4,5)P₃ formation during yeast growth and during germ tube formation

C. albicans yeast cells inoculated into fresh growth medium showed an increase in cellular Ins(1,4,5)P₃ production over a 5 h incubation period, this response varying under different growth conditions (Fig. 1). A transient increase in the Ins(1,4,5)P₃ content of yeast cells was detected between 3 and 4 h incubation, with a maximum increase of 8.5-fold to a level of 6.8 pmol per 10⁸ cells in yeast cells growing at pH 4.5 and incubated

Fig. 1. Changes in cellular Ins(1,4,5)P₃ during yeast cell growth and germ tube formation of *C. albicans*. (a) Levels of Ins(1,4,5)P₃ in yeast cells of *C. albicans* grown in defined medium at pH 4.5 at 25 °C (●) and 37 °C (○). (b) Levels of Ins(1,4,5)P₃ in *C. albicans* grown in defined medium at pH 6.7 at 37 °C (○, germ-tube-forming conditions) and 25 °C (●, yeast cell growth). Values shown are means of at least two replicates which did not differ by more than 5%; typical results are shown from one of at least two experiments, all of which gave similar results.

at 25 °C. Similar changes in cellular Ins(1,4,5)P₃ levels occurred with yeast cells growing at pH 4.5 and 37 °C, with a marked increase after 2 h incubation (Fig. 1a). The Ins(1,4,5)P₃ content of cells growing in germ-tube-inducing conditions (pH 6.7, 37 °C) increased from the start up to eight times the initial value to a level of 6.3 pmol per 10⁸ cells within 1.5 h; a level of 2.5 pmol

Fig. 2. Germ tube formation by *C. albicans* during incubation in liquid growth medium, pH 6.7, at (○) 37 °C and (●) 25 °C. Bars indicate SEM (three replicates).

per 10⁸ cells was attained in cells maintained under yeast-inducing conditions (pH 4.5, 25 °C). The initial increase of cellular Ins(1,4,5)P₃ in germ-tube-inducing conditions (Fig. 1b) occurred prior to the microscopically visible onset of germ tube formation, observed between 1 and 2 h incubation (Fig. 2). The maximum level of Ins(1,4,5)P₃, observed after 3 h, in a cell population consisting of approximately 58 % germ tubes (Fig. 2) was approximately 9 pmol per 10⁸ cells (Fig. 1b).

Phosphorylation of Ins(1,4,5)P₃

Cell extracts of *C. albicans* showed an ATP-dependent conversion of Ins(1,4,5)P₃ into InsP₄ (Fig. 3). Further phosphorylation was also observed, eluting in the 2.0 M ammonium formate/0.1 M formic acid fraction which contained InsP₅ and/or InsP₆. This suggested the presence of a kinase capable of phosphorylating the InsP₄ to InsP_{5/6}. For each cell extract the amount of [³H]Ins(1,4,5)P₃ remaining after 1 h incubation together with the amount of InsP₄ and InsP_{5/6} produced almost corresponded to the initial level of [³H]Ins(1,4,5)P₃ in the reaction mixture, indicating a sequence of direct phosphorylations. Maximum phosphorylation of Ins(1,4,5)P₃ occurred in cells grown for 5 h (Fig. 3a, b parts iii and iv). After incubation for 60 min, phosphorylation of Ins(1,4,5)P₃ in extracts of cells grown for 1.5 h in control (yeast cell) and germ-tube-inducing conditions was similar, with between 16 and 26 % of the ³H-labelled substrate being converted to InsP₄ (Fig. 3a parts i and ii). The maximum production of InsP_{5/6} (approximately 5 %) occurred in extracts from cells grown at pH 6.7 and 37 °C for 1.5 h (Fig. 3a part ii),

Fig. 3. (a) ATP-dependent phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to InsP_4 and $\text{InsP}_{5/6}$ by cell extracts of *C. albicans* grown at: pH 6.7 and 25 °C for (i) 1.5 h and (iii) 5 h; pH 6.7 and 37 °C for (ii) 1.5 h and (iv) 5 h. (b) ATP-dependent phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to InsP_4 and $\text{InsP}_{5/6}$ by cell extracts of *C. albicans* grown at: pH 4.5 and 25 °C for (i) 1.5 h and (iii) 5 h; pH 4.5 and 37 °C for (ii) 1.5 h and (iv) 5 h. Extracts were analysed as described in Methods. $\circ$, InsP_3 ; $\bullet$, InsP_4 ; $\square$, $\text{InsP}_{5/6}$. Values are expressed as radioactivity (d.p.m.) per 10^8 cells. Mean values are shown with the SEM where this exceeds the dimensions of the symbols.

which corresponded to the period of initial germ tube formation (Fig. 2). Extracts from cells grown for 5 h at pH 4.5 and 25 °C (Fig. 3b part iii) and at pH 6.7 and 37 °C (Fig. 3a part iv) demonstrated approximately 30% conversion of $\text{Ins}(1,4,5)\text{P}_3$ to InsP_4 with further phosphorylation (around 7–10%) to $\text{InsP}_{5/6}$. Maximum production of InsP_4 (approximately 40%) from $\text{Ins}(1,4,5)\text{P}_3$ was measured in extracts from cells of *C. albicans* grown for 5 h at pH 4.5 and 37 °C (Fig. 3b part iv) and pH 6.7 and 25 °C (Fig. 3a part iii), both of these growth conditions supporting yeast-like growth of the

organism. At pH 4.5 and 37 °C, 26% of the original substrate was converted to $\text{InsP}_{5/6}$, while cells grown at pH 6.7 and 25 °C converted less substrate (15%) to the more phosphorylated products.

Kinetic determinations of $\text{Ins}(1,4,5)\text{P}_3$ kinase and phosphatase

Fig. 4(a) shows dependence of the activity of $\text{Ins}(1,4,5)\text{P}_3$ kinase on substrate concentration together with a Woolf plot of the data, used to derive the affinity constant. The

Fig. 4. Kinetic analyses of Ins(1,4,5)P₃ kinase and phosphatase from *C. albicans*. (a) Dependence of Ins(1,4,5)P₃ kinase activity on substrate concentration. Soluble cell extracts of *C. albicans* (yeast cells, grown for 5 h at pH 6.7 and 25 °C) were incubated with various concentrations of Ins(1,4,5)P₃ and 10 mM ATP and the initial rate of InsP₄ formation was determined as described in Methods. (b) Dependence of Ins(1,4,5)P₃ phosphatase activity on substrate concentration. Particulate cell extracts of *C. albicans* (yeast cells, grown for 5 h at pH 6.7 and 25 °C) were incubated with various concentrations of Ins(1,4,5)P₃ and 10 mM ATP and the initial rate of InsP₂ formation was determined as described in Methods. The insets show Woolf plots of the data. Data shown are mean values from one of at least two experiments, giving similar results.

apparent saturation concentration of substrate is approximately 25 μM (Fig. 4a), while the calculated K_m value of Ins(1,4,5)P₃ kinase for Ins(1,4,5)P₃ of 9 μM, derived from Fig. 4(a), is around apparent physiological levels of this compound in *C. albicans*, as calculated using approximate cell volume (47 fl) and Ins(1,4,5)P₃ determination values. The capacity of *C. albicans* to convert Ins(1,4,5)P₃ to InsP₄ confirms that these cells possess an active InsP₃ kinase. However, the apparent K_m value of Ins(1,4,5)P₃ phosphatase for Ins(1,4,5)P₃, in particulate cell extracts, of approximately 480 μM (derived from Fig. 4b) is greater than the cellular concentration of Ins(1,4,5)P₃. A similar K_m value was obtained for Ins(1,4,5)P₃ phosphatase from soluble cell extracts (data not shown). Such apparent low affinity of this enzyme for the substrate might indicate the presence

of a non-specific phosphatase in cell extracts, although the use of Ins(1,4,5)P₃ phosphatase inhibitors (Hansbro *et al.*, 1994) would be necessary to confirm this. However, the very slow rate of dephosphorylation of Ins(1,4,5)P₃ to InsP₂ would suggest that the importance of Ins(1,4,5)P₃ phosphatase within the cell is considerably less than that of the Ins(1,4,5)P₃ kinase.

Ins(1,4,5)P₃ kinase activities

When cell extracts of *C. albicans* were incubated in the presence of 10 mM ATP and Ins(1,4,5)P₃ (at concentrations in excess of the K_m value), the specific activities of Ins(1,4,5)P₃ kinase were approximately two to threefold greater in yeast cells and germ tubes grown for 5 h than in cultures grown for 1.5 h (Table 1). In cells grown for 1.5 and 5 h at pH 4.5 and 25 °C (yeast-like growth), the specific activities of the enzyme (30 and 88 pmol min⁻¹ per 10⁸ cells, respectively) were lower than the activities from cells grown in other yeast- and germ-tube-inducing conditions. At the onset of germination, 1.5 h incubation at pH 6.7 and 37 °C, the specific activity of the enzyme (51 pmol min⁻¹ per 10⁸ cells) was slightly higher than in yeast-like cells. At substrate concentrations of approximately 0.1 of the K_m value (0.9 μM) and in the presence of 10 mM ATP, the maximum level of Ins(1,4,5)P₃ kinase activity was observed in extracts from 5 h cultures of yeast-like cells and germ tubes (Table 1). This increase corresponded to the elevated levels of InsP₄ detected in cell extracts from the same incubation times (Fig. 3). The level of Ins(1,4,5)P₃ kinase activity of 6 pmol min⁻¹ per 10⁸ cells in cells grown for 1.5 h at pH 6.7 and 37 °C (germ-tube-inducing conditions) was higher than the activity observed for cells growing in yeast-like conditions, with the exception of cells grown for 1.5 h at pH 4.5 and 25 °C (7 pmol min⁻¹ per 10⁸ cells).

DISCUSSION

In eukaryotes, Ins(1,4,5)P₃ is metabolized by two principal routes catalysed by Ins(1,4,5)P₃ 5-phosphatase (phosphomonoesterase) and Ins(1,4,5)P₃ 3-kinase, with the relative balance between the two routes dependent on regulatory conditions (Estevez *et al.*, 1994). The kinase pathway is particularly important as a feedback control point in Ins(1,4,5)P₃ metabolism because activity is stimulated by Ca²⁺/CaM (Estevez *et al.*, 1994). A Ca²⁺/CaM involvement in the dimorphic transition of *C. albicans* has been established previously (Hubbard *et al.*, 1982; Sabie & Gadd, 1989). However, work with *S. cerevisiae* has found that the yeast Ins(1,4,5)P₃ kinase differs from known mammalian enzymes, and appears to be an Ins(1,4,5)P₃ 6-kinase, not a 3-kinase, and insensitive to Ca²⁺/CaM in the purified state (Estevez *et al.*, 1994). Since yeast cells and germ tubes of *C. albicans* rapidly phosphorylated Ins(1,4,5)P₃ to InsP₄ and InsP_{5/6}, with very low levels of dephosphorylation occurring, it is suggested that Ins(1,4,5)P₃ has an important role as a precursor for the production of these compounds. The

Table 1. Ins(1,4,5)P₃ kinase activity of *C. albicans*

Specific activity of Ins(1,4,5)P₃ kinase was determined in cell extracts of *C. albicans* incubated under varying conditions of pH and temperature to promote yeast cell growth (pH 4.5, 25 °C; pH 6.7, 25 °C; pH 4.5, 37 °C) or germ tube formation (pH 6.7, 37 °C). Cell extracts were incubated with Ins(1,4,5)P₃ at concentrations in excess of, or approximately 0.1 of, the K_m value as described in Methods. Values shown are means $\pm$ SEM.

Growth conditions			Ins(1,4,5)P ₃ kinase activity (pmol min ⁻¹ per 10 ⁸ cells)	
Incubation time (h)	pH	Temp. (°C)	[Ins(1,4,5)P ₃] = 0.1 K_m	[Ins(1,4,5)P ₃] > K_m
1.5	4.5	25	6.6 $\pm$ 0.9	30.0 $\pm$ 2.7
1.5	6.7	25	3.8 $\pm$ 0.8	49.5 $\pm$ 1.0
5.0	4.5	25	12.4 $\pm$ 1.1	88.4 $\pm$ 4.2
5.0	6.7	25	6.8 $\pm$ 0.6	148.6 $\pm$ 6.3
1.5	4.5	37	4.7 $\pm$ 0.9	45.0 $\pm$ 3.6
1.5	6.7	37	6.0 $\pm$ 1.2	50.6 $\pm$ 1.7
5.0	4.5	37	10.5 $\pm$ 0.8	116.5 $\pm$ 13.0
5.0	6.7	37	7.7 $\pm$ 0.5	110.4 $\pm$ 3.2

accumulation of InsP₄ in *C. albicans* may also indicate other important physiological functions mediated by this compound. Indeed, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄] is also a postulated intracellular signalling molecule which may act with Ins(1,4,5)P₃ in the regulation of [Ca²⁺]_i by influencing Ca²⁺ influx (Irvine, 1992; Menniti *et al.*, 1993). An association between Ca²⁺ influx and signal transduction in *S. cerevisiae* (Iida *et al.*, 1990) and the dimorphic fungus *O. ulmi* (Brunton & Gadd, 1991; Gadd & Brunton, 1992) has been postulated. Ins(1,3,4,5)P₄ is also linked to the further synthesis of inositol polyphosphates, e.g. InsP₅ and InsP₆ (Menniti *et al.*, 1993). The presence of InsP₄, InsP₅ and InsP₆ has been reported in *N. crassa* (Lakin-Thomas, 1993b), with the levels of these inositol polyphosphates increasing during inositol starvation. It has been well documented that during germination plant seeds mobilize InsP₆ (Gibson & Ullah, 1990) and Graf *et al.* (1984) have suggested that inositol polyphosphates can inhibit iron-catalysed hydroxyl radical formation.

The K_m value of Ins(1,4,5)P₃ kinase (approx. 9 μ M) in *C. albicans* is similar to the K_m value of 7 μ M reported for *S. cerevisiae* (Estevez *et al.*, 1994). However, Ins(1,4,5)P₃ phosphatase from *C. albicans* appeared to have a very low affinity for Ins(1,4,5)P₃. The amount of substrate required to achieve maximum velocity of the enzyme exceeded physiological concentrations of substrate in the cell, suggesting that this enzyme is physiologically less important than Ins(1,4,5)P₃ kinase. Moreover, Ins(1,4,5)P₃ phosphatase from other eukaryotic sources seems to be a relatively non-specific enzyme recognizing both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ as substrates (Lampe *et al.*, 1994). It is relevant that work carried out on *S. cerevisiae* has described a lack of detectable monophosphatase activity. Indeed, no InsP₂, inositol mono-

phosphate or free inositol was detected during incubation of cell extracts with Ins(1,4,5)P₃ as substrate, even under conditions where possible effects due to proteolysis, dephosphorylation and thiol groups were limited (Estevez *et al.*, 1994). However, inositol tris-, bis- and monophosphatases were detected in *N. crassa* with the inositol bis- and monophosphatases exhibiting reduced activity in the presence of LiCl (Hanson, 1991). A similar response to LiCl has been described for mammalian systems, the main target for this compound appearing to be inhibition of monophosphatase activity (Berridge & Irvine, 1989; Sillence & Downes, 1992; Gani *et al.*, 1993).

As well as a role as a precursor of inositol polyphosphates, Ins(1,4,5)P₃ may also act as a second messenger in a signal transduction pathway involving the release of Ca²⁺ from intracellular stores (see Gadd, 1995). In contrast to Ins(1,4,5)P₃, other intracellular second messengers, including Ca²⁺ [which may be released from intracellular stores by the action of Ins(1,4,5)P₃] and cAMP, have been more readily implicated in the morphological transitions of a range of fungi but it should be noted that all three second messengers and associated pathways may interact in eukaryotic organisms (Nishizuka, 1984; Gadd, 1995). The proposed involvement of Ca²⁺ during the yeast-mycelium transition of some dimorphic fungi has been well documented (Muthukumar & Nickerson, 1984; Muthukumar *et al.*, 1986; Brunton & Gadd, 1991; Gadd & Brunton, 1992; Sabie & Gadd, 1989). Similarly, much information exists on the involvement of cAMP during the dimorphic transition of *C. tropicalis* (Omi & Kamihara, 1989), *C. albicans* (Sabie & Gadd, 1992), *Aureobasidium pullulans* (Cooper *et al.*, 1985) and *O. ulmi* (Brunton & Gadd, 1989). Exogenous cAMP was found to increase branching in *F. graminearum* (Robson

et al., 1991b). cAMP exerts its effect by binding to and regulating cAMP-dependent protein kinases (Pall, 1981; Gancedo *et al.*, 1985; Cohen & Hardie, 1991). Kato *et al.* (1989) have demonstrated PtdIns kinase stimulation by cAMP-dependent protein kinase activity in *S. cerevisiae*, which may result in the activation of other signalling components such as Ins(1,4,5)P₃ and DAG. Thus, an increase in Ins(1,4,5)P₃ levels was reported during nitrogen stimulation in *S. cerevisiae* (Schomerus & Kuntzel, 1992) and during ethanol-induced filamentous growth of *C. tropicalis*, enhanced PtdIns metabolism was observed (Uejima *et al.*, 1987). However, work by Lakin-Thomas (1993a) did not detect a phosphoinositide signalling system in blue-light-induced phase resetting in *N. crassa*. Similarly, during various stimulus-response systems, e.g. light, choline or glucose derepression, investigated in *F. graminearum* and *N. crassa*, a phosphoinositide signalling system was not observed (Prior *et al.*, 1994). Some of these observations in different fungi may imply the existence of alternative signalling mechanisms in the different fungal species, although some difficulties in analytical procedures, particularly with heterogeneous filamentous fungal biomass, may make the detection of short-term changes in proposed second messengers difficult. It was thought that an organism like *C. albicans* which can exhibit dimorphism between two major cellular forms, yeast cells and hyphae, would provide a more easily manipulated model to investigate changes in Ins(1,4,5)P₃ levels and metabolism in the different cell types and during the yeast-hyphal transition. Indeed, the major change observed when yeast cells were stimulated to produce germ tubes at 37 °C was a marked increase in Ins(1,4,5)P₃, in comparison with yeast cells incubated at pH 4.5 at 37 °C. Such an increase preceded and paralleled germ tube formation and was particularly marked over the first 2 h. Such an observation does implicate a potential role for phosphoinositide turnover in germ tube formation. However, despite this and the observation that the maximum increase in cellular Ins(1,4,5)P₃ during yeast cell growth was not as great as that observed during germ tube formation, it is difficult to speculate whether a threshold level of Ins(1,4,5)P₃ is required to induce germ tubes. The potential multiplicity of signal-dependent processes in eukaryotic cell development impedes dissection (O'Day, 1990) and, indeed, a transient increase of Ins(1,4,5)P₃ was observed during yeast-like growth of *C. albicans* which corresponded to the period of cell evagination as described by Buffo *et al.* (1984). Such an increase may be accounted for by various physiological processes occurring in the cell during budding, including DNA, RNA and protein synthesis (Brummel & Soll, 1982) and the regulation of nuclear migration and division (Bedell *et al.*, 1980). If the existence of an Ins(1,4,5)P₃-mediated Ca²⁺-signalling system is postulated, then this could also be involved in those processes concerned with yeast cell growth.

It is relevant that the vacuolar membranes of *C. albicans* have been shown to possess two Ca²⁺-release pathways, one induced by Ins(1,4,5)P₃ and the other by an inside-

positive voltage. The Ins(1,4,5)P₃-gated mechanism exhibits a K_{0.5} of 2.4 µM for Ins(1,4,5)P₃ but was not activated by inositol 4,5-bisphosphate or Ins(1,3,4,5)P₄ (Calvert & Sanders, 1995). Both Ca²⁺-release pathways may act independently with voltage-sensitive release being controlled by desensitization and with Ins(1,4,5)P₃-induced Ca²⁺ release controlled by the presence of Ins(1,4,5)P₃. Both mechanisms for vacuolar Ca²⁺ release may be affected by [Ca²⁺]_o so that Ins(1,4,5)P₃-induced Ca²⁺ release may stimulate further [Ca²⁺]_o-induced Ca²⁺ release (Calvert & Sanders, 1995). An Ins(1,4,5)P₃-gated channel has also been reported on the vacuolar membranes of *N. crassa* (Cornelius *et al.*, 1989) and *S. cerevisiae* (Belde *et al.*, 1993) although Ca²⁺ was not released from intracellular fractions or vacuoles of *Penicillium notatum*, an organism where exogenous Ca²⁺ induces sporulation and CaM-mediated phosphorylation (Pitt & Barnes, 1993). In *C. albicans*, some physiological signals which may trigger Ca²⁺ release include cytoplasmic alkalization (Stewart *et al.*, 1988), which could stimulate voltage-sensitive Ca²⁺ release from the vacuole (Bertl & Slayman, 1992; Calvert & Sanders, 1995), and elevation of cellular cAMP, which can occur during the yeast-mycelium transition (Sabie & Gadd, 1992); cAMP can stimulate Ins(1,4,5)P₃ production because of its action on the enzymes of Ins(1,4,5)P₃ synthesis (Kato *et al.*, 1989). Mobilized Ca²⁺ may act through CaM, which is present in *C. albicans* (Hubbard *et al.*, 1982) and involved in the dimorphic transition (Sabie & Gadd, 1989), thus stimulating protein phosphorylation (Paranjape & Datta, 1990; Paranjape *et al.*, 1990). Thus, the basic requirements of an Ins(1,4,5)P₃-mediated signalling system are now known to exist in *C. albicans* and a simple model showing the relationship between Ins(1,4,5)P₃ and its metabolism, intracellular Ca²⁺ stores, transport processes and cAMP is shown in Fig. 5. However, further work is clearly needed for detailed dissection of the multiplicity and overlap of the signalling processes that may occur and their regulation; an important role for Ca²⁺/CaM-dependent protein phosphatases can also be envisaged as in *S. cerevisiae* (Cunningham & Fink, 1994).

Although the fungal vacuole is recognized as the prime intracellular store for calcium, and undoubtedly plays a significant role in cytosolic ion and pH homeostasis, the possible involvement of the Golgi apparatus, endoplasmic reticulum and mitochondria in Ca²⁺ signalling awaits definite confirmation, particularly at a physiological level (Okorokov, 1994). In addition, analysis of the key enzymes of Ins(1,4,5)P₃ metabolism has suggested that Ins(1,4,5)P₃ may function predominantly as an essential precursor for synthesis of inositol polyphosphates such as InsP₅, as proposed for *S. cerevisiae* (Estevez *et al.*, 1994). The role(s) of such polyphosphates in fungal metabolism (including phosphorus storage) and signal transduction by modulation of ion fluxes at plasma and organellar membranes is largely unknown at present. This is perhaps not surprising; Prior *et al.* (1993) have demonstrated a

Fig. 5. Outline model of the interrelationship between the three important second messengers cAMP, Ca^{2+} and $\text{Ins}(1,4,5)\text{P}_3$ in *C. albicans*. $[\text{Ca}^{2+}]_c$, free cytosolic Ca^{2+} , and $[\text{Ca}^{2+}]_v$, free vacuolar Ca^{2+} , are shown as parts of the total cytosolic/vacuolar pool, respectively. The relationship of other Ca^{2+} pools (e.g. Golgi, endoplasmic reticulum, mitochondria) to the total Ca^{2+} pool, and the stimulatory effect of InsP_4 on Ca^{2+} influx, are depicted as uncertain (?). Transport processes involved in accumulation and intracellular compartmentation of cell Ca^{2+} are shown at the plasma membrane [H^+ -ATPase, $\text{Ca}^{2+}/\text{H}^+$ antiport (efflux), Ca^{2+} channel (influx)] and vacuolar membrane [H^+ -ATPase, $\text{Ca}^{2+}/\text{H}^+$ antiport and Ca^{2+} -ATPase (influx); $\text{Ins}(1,4,5)\text{P}_3$ - and voltage-sensitive Ca^{2+} channels (efflux)]. $[\text{Ca}^{2+}]_c$ is depicted as acting through CaM with the overall cellular response determined by phosphorylation/dephosphorylation reactions. +, Positive/stimulatory effects. $\text{Ins}(1,4)\text{P}_2$, inositol 1,4-bisphosphate; PKC, protein kinase C; $\text{PtdIns}(3)\text{P}$, phosphatidylinositol 3-phosphate; $\text{PtdIns}(4)\text{P}$, phosphatidylinositol 4-phosphate. Information was derived from several sources, including Kato et al. (1989), Sabie & Gadd (1989), Miller et al. (1990), Paranjape et al. (1990), Calvert & Sanders (1995) and Gadd (1995).

complexity in the number of isomers of InsP_2 and InsP_3 in fungi that has not been observed in animal or plant cells. Further work with an easily manipulated model organism such as *C. albicans*, which shows distinct phase transitions in response to defined environmental stimuli, may prove profitable, particularly in view of the advances that have been made in the dissection of this organism at subcellular and molecular levels (Gow, 1994, 1995; Calvert & Sanders, 1995).

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