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Involvement of calcium, calmodulin and protein phosphorylation in morphogenesis of *Candida albicans*

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N-Acetyl-D-glucosamine-induced germ tube formation in *Candida albicans* at 37 °C was accompanied by an increase in the rate of protein phosphorylation. The calmodulin antagonist trifluoperazine and the Ca^{2+} ionophore A23187, which inhibited germ tube formation, also reduced the rate of phosphorylation. The rate of phosphorylation was also reduced when cells were incubated at 25 °C, which favoured yeast-phase growth. Two-dimensional SDS-PAGE analysis of phosphoproteins from germ-tube-forming and yeast cells revealed two germ-tube-specific and three yeast-specific phosphoproteins. Germ tubes and hyphae had more calmodulin activity than yeast cells, irrespective of the germ-tube-inducing condition used. As a first step towards understanding the inhibitory effect of trifluoperazine on germ tube formation, calmodulin from *C. albicans* was purified to homogeneity. It was heat stable, and displayed a pronounced Ca^{2+}-induced shift in electrophoretic mobility.

Introduction

Candida albicans is a widespread opportunistic fungal pathogen that grows either as a yeast or as filamentous hyphae, depending on the environmental conditions (Odds, 1985, 1988). A germ tube is an intermediate stage in the yeast-to-hypha transition which can be induced by a number of factors. Morphogenesis in *C. albicans* has attracted particular attention since it seems to be relevant to the virulence of the organism. It also provides a good model system for studying eukaryotic cell differentiation. Several experimental approaches have been applied to the investigation of morphogenesis of *C. albicans*, but the underlying mechanism is still not clear (Shepherd *et al.*, 1985; Odds, 1988; Datta *et al.*, 1989).

Ca^{2+} is a second messenger (Carafoli, 1987) whose concentration regulates the activity of many protein kinases (Greengard, 1978). Protein kinase action is a primary mechanism for transduction of extracellular stimuli. The action of Ca^{2+} is mediated mainly through calmodulin, a calcium-binding protein that regulates many cellular processes in eukaryotes (Cheung, 1980; Means and Dedman, 1980). Calmodulin has been detected in many yeasts and filamentous fungi (Hubbard *et al.*, 1982; Muthukumar *et al.*, 1987), but its precise role in yeast in relation to Ca^{2+} is not known. However, several lines of investigation suggest that Ca^{2+} is an important regulatory ion in yeast (Davis *et al.*, 1986). The presence of calmodulin and of calmodulin-dependent protein kinases in *Saccharomyces cerevisiae* (Hubbard *et al.*, 1982; Miyakawa *et al.*, 1989) and *Neurospora crassa* (Ortega Perez *et al.*, 1981; Tuinen *et al.*, 1984) indicates that calmodulin has a regulatory role in fungi.

The activator function of Ca^{2+}-calmodulin is antagonized by a variety of pharmacological agents, including phenothiazine derivatives such as trifluoperazine (TFP) (Weiss *et al.*, 1980). Accordingly, inhibition of specific physiological processes by such calmodulin antagonists has provided the evidence for the possible involvement of Ca^{2+}-calmodulin in such processes. However, calmodulin is not the only site of action of these calmodulin antagonists, as TFP is known to inhibit protein kinase C (Schatzmann *et al.*, 1981). Ca^{2+} and calmodulin have been reported to be involved in differentiation of fungi such as *Ceratocystis ulmi* (Methukumar & Nickerson, 1984), *Dictyostelium discoideum* (Lydan & O'Day, 1988) and *Physarum polycephalum* (Uyeda & Furya, 1986). Protein phosphorylation is also reported to be involved in the growth and differentiation of *Mucor* (Orlowski & Sypherd, 1978), *D. discoideum* (Sinclair & Rickwood, 1985) and *P. polycephalum* (Fronk & Toczko, 1987). It has been reported recently that Ca^{2+} and calmodulin-mediated protein phosphorylation play a role in germination and growth of *Metarhizium anisopliae* (St Leger *et al.*, 1989).

We reported earlier that TFP blocks morphogenesis in *C. albicans* (Gupta Roy & Datta, 1987). In this report, we

Abbreviation: TFP, trifluoperazine.

have purified calmodulin from *C. albicans* and have shown that Ca^{2+} and calmodulin may regulate morphogenesis in *C. albicans* by differential protein phosphorylation.

Methods

Organism and growth conditions. *C. albicans* ATCC 10261 was maintained on a medium containing 2% peptone, 1% yeast extract, 2% glucose and 2% agar (all w/v). The cells were grown for 17 h in a medium containing 1% glucose, 0·5% peptone and 0·3% KH_2PO_4 (GPK) and then transferred to a new medium (with half the concentration of glucose) and grown for 11 h into stationary phase.

Induction of germ tubes or hyphae. Germ tubes were induced as described elsewhere (Shepherd *et al.*, 1980; Natarajan *et al.*, 1984). Approximately 5×10^7 cells ml^{-1} were incubated at 37 °C in a 20 mM-imidazole/HCl buffer (pH 6·6) containing 0·2 mM-$MnCl_2$ and 5 mM-*N*-acetyl-D-glucosamine (GlcNAc) as inducer. Germ tube formation was inhibited either by addition of 20 μM-TFP or calcium ionophore A23187 (4 μM) to the induction medium or by incubating cells at 25 °C. For some experiments, an amino acid synthetic medium (Lee *et al.*, 1975) at pH 6·8 was used to induce germ tube formation at 37 °C.

Calmodulin purification. Calmodulin was purified by modification of methods used to purify calmodulin from bovine brain (Gopalakrishna & Anderson, 1982) and *S. cerevisiae* (Davis *et al.*, 1986). *C. albicans* was cultured in GPK medium at 30 °C for 15 h in a fermenter (Bioengineering). Cells (80 g wet weight) were suspended in 80 ml of homogenization buffer (50 mM-Tris, pH 7·5, 1 mM-EDTA and 1 mM-phenylmethylsulphonyl fluoride (PMSF). Glass beads (80 g; 0·45 mm) were added and the cells were lysed by four 30 s pulses in a cell homogenizer (Braun) in four batches. About 90% of the cells were broken. The lysate was centrifuged at 12000 *g* for 30 min; the supernatant was incubated in a boiling water bath for 3 min and then cooled in ethanol/ice bath for 10 min. Precipitated material was removed by centrifugation at 12000 *g* for 30 min. The pH of the supernatant was then adjusted to 7·5 with Tris base, $CaCl_2$ was added to give a final concentration of 5 mM, and the mixture was loaded on a column (25 ml) of phenyl-Sepharose (Pharmacia) at room temperature. The column was washed with a solution containing 50 mM-Tris/HCl, pH 7·5, 0·1 mM-$CaCl_2$ and 1 mM-PMSF and then with the same buffer solution containing 0·5 M-NaCl. Calmodulin was eluted with a buffer containing 50 mM-Tris/HCl, pH 7·5, 1 mM-PMSF and 7·5 mM-EGTA. A single peak fraction was collected and dialysed against water at 4 °C. The dialysed sample was lyophilized, dissolved in 10 mM-Tris/HCl, pH 7·5, and then passed through a membrane filter (30 kDa cut-off), using an ultrafiltration cell (Amicon). The filtrate contained calmodulin. To identify the protein as calmodulin, a sample (1 μg) was subjected to SDS-PAGE in the presence of either $CaCl_2$ (10 mM) or EGTA (10 mM) as described by Davis *et al.* (1986).

Calmodulin assay. Germ tubes/hyphae and yeast cells were harvested from 5 ml induction medium after 3 h incubation. Cells were washed with sterile water, and the pellet was suspended in 0·2 ml homogenization buffer (10 mM-Tris/HCl, pH 7·5, 0·5-mM-PMSF). Cells were then broken by mechanical disruption with glass beads (0·45 mm) and centrifuged at 12000 *g* for 3 min. The supernatant was incubated in a boiling water bath for 3 min, cooled in an ethanol/ice bath, and centrifuged at 12000 *g* for a further 3 min. The resulting supernatant was assayed for calmodulin activity by its capacity to stimulate cAMP-phosphodiesterase, using the method of Dedman & Means (1977). Calmodulin activity was expressed as nmol cAMP hydrolysed min^{-1} $(mg\ protein)^{-1}$. This experiment was repeated three times; data from a representative experiment are shown.

Rate of ^{32}P incorporation into proteins. Cells (5×10^7 cells ml^{-1}) were suspended in imidazole-buffered medium with GlcNAc (5 mM), and incubated at 37 °C. TFP (20 μM) or A23187 (4 μM) was added to the cell suspension at 0 h. $CaCl_2$ (10 mM) was added after 2·5 h incubation in the presence of A23187. The rate of ^{32}P incorporation was measured as described previously (Gupta Roy & Datta, 1987).

Radioactive labelling for phosphoprotein analysis. Cells (5×10^7 cells ml^{-1}) in imidazole-buffered GlcNAc (5 mM) medium were incubated at 37 °C in a rotary shaker at 200 r.p.m. Germ tube formation was blocked either by addition of TFP (20 μM) or by incubating cells at 25 °C. Cells were labelled with $H_3{}^{32}PO_4$ (250 μCi ml^{-1}) for 3 h, harvested, and washed with ice-cold 5 mM-sodium phosphate. The final pellet was suspended in homogenization buffer containing 10 mM-Tris/HCl, pH 6·8, 0·5 mM-PMSF, RNAase (50 μg ml^{-1}) and DNAase (50 μg ml^{-1}). Glass beads (0·45 mm) were added and cells were broken by five 1 min periods of vortexing, each interspersed with 1 min in an ice bath. Glass beads, unbroken cells and cell debris were removed by centrifugation at 12000 g for 10 min at 4 °C.

Gel electrophoresis. Cell extracts were lyophilized, dissolved in 30 μl IEF sample buffer (O'Farrell, 1975) and subjected to non-equilibrium pH-gradient electrophoresis (O'Farrell *et al.*, 1977). Proteins were separated by isoelectric focusing in tube gels in the first dimension with 5% ampholytes, pH 3–10 (Pharmacia). The second dimension was run in slab gels containing 10·5% (w/v) polyacrylamide using the buffer system described by Laemmli (1970). After electrophoresis, the gels were treated for 30 min with 16% (w/v) TCA at 95 °C as described by Mannai & Cozzone (1982). The gels were finally stained, destained and dried. Dried gels were exposed to Kodak X-Omat AR film at −70 °C with intensifying screens for autoradiography. Gels were loaded with equal amounts of TCA-precipitable radioactivity. Standard proteins (Pharmacia) were run with every second-dimension gel. These experiments have been repeated more than five times. All results were taken into account and only the changes that were consistent are indicated in the figures.

Results

Effect of trifluoperazone (TFP) and calcium ionophore (A23187) on germ tube formation and growth of C. albicans

We had earlier reported that TFP, a calmodulin inhibitor, blocks germ tube formation in *C. albicans* without affecting normal growth at 30 °C (Gupta Roy & Datta, 1987). To confirm that TFP is not toxic to cells at the concentration used, its effect on *C. albicans* growth was studied. There was no effect on growth at 37 °C, a temperature at which germ tube formation was blocked in the presence of TFP (Fig. 1). In the amino acid synthetic medium (Lee *et al.*, 1975), at pH 6·8, cells form only hyphae when incubated at 37 °C. Addition of TFP (20 μM) not only blocked hypha formation but caused cells to grow as yeasts (data not shown). This supports the earlier observation that TFP specifically inhibits germ tube/hypha formation but not the growth of the organism. Calcium ionophore A23187 also blocked germ tube formation, an effect which was reversed by calcium

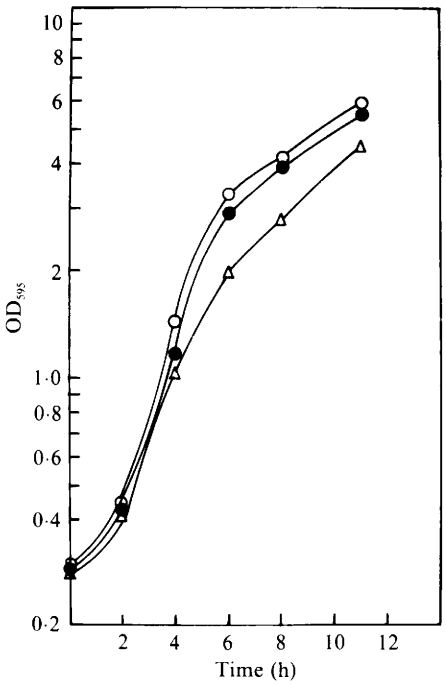

Fig. 1. Effect of TFP and A23187 on growth of *C. albicans* in medium containing 0·5% glucose, 0·5% peptone and 0·3% KH_2PO_4 at 37 °C. ○, Normal growth; ●, in presence of TFP (20 μM); △, in presence of calcium ionophore A23187 (4 μM).

(Gupta Roy & Datta, 1987), but A23187 had no significant effect on growth (Fig. 1). In a further test, cells were incubated in the presence of A23187 for 2·5 h. $CaCl_2$ (10 mM) was then added, and incubation continued for 3 h. About 70% of the cells were able to form germ tubes (data not shown). Since TFP is a calmodulin inhibitor, we checked calmodulin activity in germ-tube-forming and yeast cells (Table 1). Germ tubes or hyphae formed in imidazole-buffered GlcNAc medium or in amino acid synthetic medium had more calmodulin activity than yeast cells.

Calmodulin purification

In order to confirm the presence of calmodulin in *C. albicans*, we purified it to homogeneity. The purified protein displayed a pronounced Ca^{2+}-induced shift in electrophoretic mobility (Fig 2), which is an important criterion for identification of calmodulin (Davis *et al.*, 1986). In the presence of 10 mM-Ca^{2+}, the protein had an apparent molecular mass of 15 kDa, as judged by SDS-PAGE, whereas in the presence of EGTA (10 mM), the apparent molecular mass was 18·5 kDa. The two bands observed in the presence of Ca^{2+} were due to the limited concentration of Ca^{2+}. The ability to bind calcium is an important property of calmodulin. To demonstrate Ca^{2+} binding directly, purified calmodulin was bound to nitrocellulose membrane and incubated in a solution containing $^{45}Ca^{2+}$. After washing, autoradiography of the membrane showed that Ca^{2+} was bound to calmodulin (data not shown).

Protein phosphorylation during morphogenesis

We showed previously that germ tube formation in *C. albicans* is accompanied by an increase in the rate of ^{32}P incorporation (Gupta Roy & Datta, 1987). TFP, which blocks germ tube formation, also reduced the rate of ^{32}P incorporation, to a level comparable to that observed at 25 °C (Fig. 3*a*). At 25 °C, cells remain in the yeast form and do not form germ tubes even in the presence of GlcNAc. A23187, which also blocks germ tube formation, also reduced the rate of ^{32}P incorporation. Interestingly, both of these effects of A23187 could be reversed by addition of 10 mM-Ca^{2+} (Fig. 3*b*). This result suggests that Ca^{2+}-calmodulin-dependent phosphorylation is involved in morphogenesis in *C. albicans*.

Fig. 4 represents a two-dimensional electrophoresis gel of total phosphoproteins extracted from germ-tube-forming and yeast cells. An equal amount of TCA-precipitable radioactivity was loaded on each gel to

Table 1. *Calmodulin activity during morphogenesis of* C. albicans

Germ tube/hypha forming medium	Temperature (°C)	pH	TFP (20 μM)	Morphology	Calmodulin Sp. act.*
Imidazole-buffered, GlcNAc	37	6·6	−	Germ tube	4·3
(Shepherd *et al.*, 1980;	37	6·6	+	Yeast	1·9
Natarajan *et al.*, 1984)	25	6·6	−	Yeast	1·9
Amino acid synthetic medium	37	6·8	−	Hypha	4·3
(Lee *et al.*, 1975)	37	4·5	−	Yeast	2·2
	25	6·8	−	Yeast	2·6

* Calmodulin specific activity was expressed as nmol cAMP hydrolysed min^{-1} (mg protein)$^{-1}$. This experiment has been repeated three times. Data of a representative experiment has been shown.

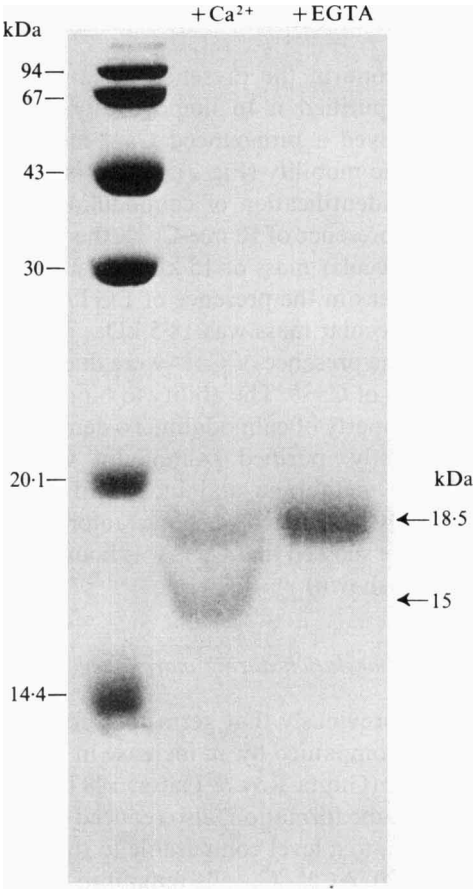

Fig. 2. Analysis of calmodulin by SDS-PAGE. Purified calmodulin (1 μg) was subjected to electrophoresis in SDS-polyacrylamide gels (14% acrylamide) in the presence of either 10 mM-$CaCl_2$ or 10 mM-EGTA. The monomer acrylamide:*N,N'*-methylenebisacrylamide ratio was 38:2.

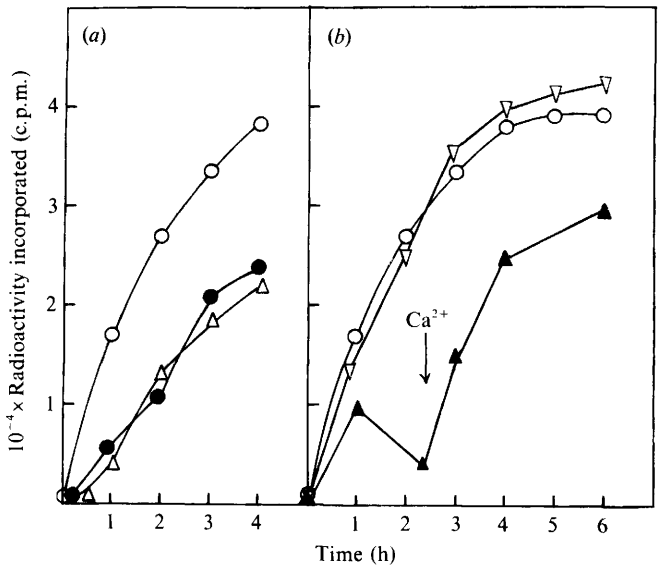

Fig. 3. Rate of ^{32}P incorporation during GlcNAc-induced germ tube formation in imidazole-buffered medium. At the indicated times, 1 ml of cells (5×10^7 cells ml^{-1}) was pulse-labelled with $H_3{}^{32}PO_4$ (0·6 μCi ml^{-1}) for 20 min at 37 °C, and hot-TCA-precipitable counts were estimated. (*a*) Effect of TFP. ○, No addition; ●, TFP (20 μM); △, incubated at 25 °C. (*b*) Effect of A23187. ○, No addition; ▽, A23187 (4 μM) plus $CaCl_2$ (10 mM); ▲, A23187 (4 μM); $CaCl_2$ (10 mM) was added at the indicated time.

reveal qualitative changes in phosphoprotein profiles of germ-tube-forming and yeast-phase cells. Fig. 4(*a*) represents total phosphoproteins extracted from germ-tube-forming cells. Fig. 4(*b*) shows the pattern obtained when 20 μM-TFP, which inhibited germ tube formation, was added to the induction medium. Fig. 4(*c*) shows the pattern obtained when cells were incubated at 25 °C, the temperature that favours yeast phase growth. Comparison of the three autoradiographs shows that the majority of the phosphoproteins resolved are common to both germ-tube-forming and yeast-phase cells. However, germ tube formation was accompanied by an increase in the level of at least two phosphoproteins (19 kDa, 17·5 kDa) (marked g in Fig. 4). Similarly, yeast-phase cells also exhibited an increase in the level of three phosphoproteins (58 kDa, 56 kDa, 44 kDa) (marked b in Fig. 4). TFP, which inhibited germ tube formation, also inhibited phosphorylation of two proteins (19 kDa, 17·5 kDa), which appear to be germ tube specific. Inhibition of the phosphorylation of these proteins could be a non-specific effect of TFP and not associated with a change in morphology. To rule out this possibility, phosphoproteins from yeast-phase cells grown in the absence of TFP, but incubated at 25 °C, were analysed (Fig. 4*c*). Under these conditions the same two proteins (19 kDa, 17·5 kDa) were not phosphorylated. The phosphorylation/dephosphorylation of these proteins is therefore associated with a change in morphology, implying that TFP inhibits germ tube formation by inhibiting phosphorylation of specific proteins. The changes in the phosphorylation/dephosphorylation status of proteins reflected the cumulative changes in the physiology of the cell as it changed morphology, rather than being associated with a single discrete event during morphogenesis.

Although comparison of autoradiographs shows other differences, only those which were consistent have been marked. The hot TCA treatment of polyacrylamide gels confirmed that we were detecting ester-linked phosphopolypeptides, since acid phosphates and acyl-linked phosphates are unstable under these conditions (Mannai & Cozzone, 1982).

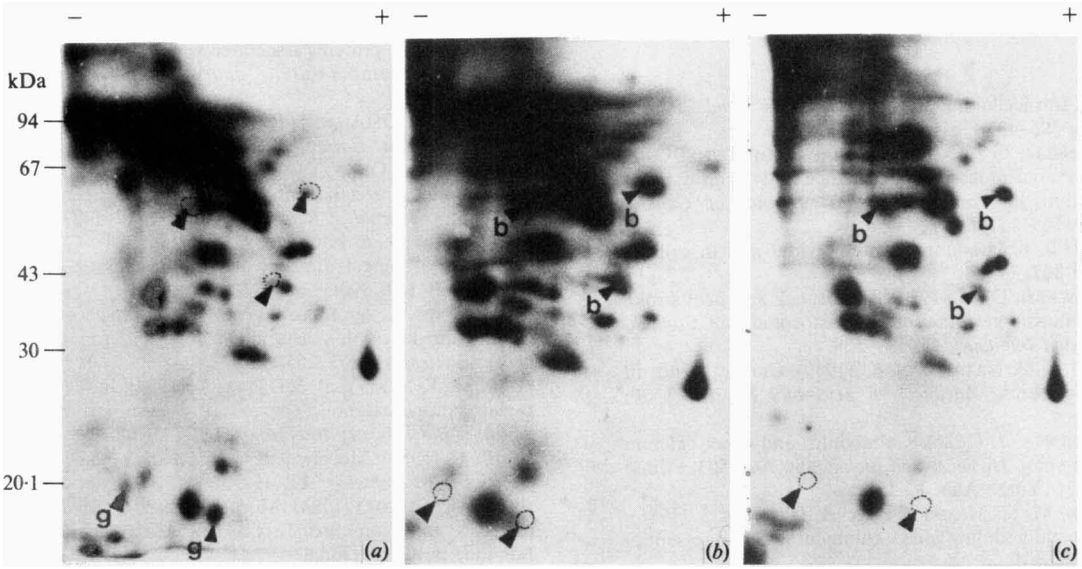

Fig. 4. Two-dimensional gel analysis of phosphoproteins from *C. albicans* during germ tube formation. Cells were labelled with $H_3{}^{32}PO_4$ for 3 h in imidazole-buffered GlcNAc medium. Samples were subjected to electrophoresis as described in Methods. (*a*) Cells incubated at 37 °C; (*b*) cells incubated at 37 °C in the presence of 20 μM-TFP; (*c*) cells incubated at 25 °C (g, germ tube specific; b, yeast specific).

Discussion

Germ tube formation induced by GlcNAc, and hypha formation in amino acid synthetic medium, are blocked by TFP, a calmodulin inhibitor, and by A23187, a calcium ionophore, without affecting the growth of the organism. TFP also blocks the germ tube formation induced by proline and glucose plus glutamine (data not shown). All these results suggest the involvement of Ca^{2+} and calmodulin in morphogenesis of *C. albicans*. Chloropromazine, a calmodulin antagonist, is also known to block germ tube formation in *C. albicans* (Gupta Roy & Datta, 1987).

A calmodulin-like protein has been reported in *C. albicans* (Hubbard *et al.*, 1982). In order to confirm its presence, we have purified calmodulin from *C. albicans*. In an attempt to identify the molecular events through which TFP exerts its effect, the calmodulin activity associated with changes in morphology was determined. Irrespective of the induction conditions used, germ-tube-forming cells had more calmodulin activity than yeast-phase cells. Morphology-associated changes in calmodulin activity could be due to changes in the intracellular levels of calmodulin or of Ca^{2+}. Interestingly, it has been reported that fungi have more calmodulin when in the filamentous form than in the yeast form (Muthukumar *et al.*, 1987). Although TFP is not a specific inhibitor of calmodulin (Schatzmann *et al.*, 1981), our results indicate that TFP probably blocks germ tube formation by inhibiting calmodulin activity. It has been suggested that cytoplasmic alkalinization accompanying germ tube formation can mediate morphogenesis by altering calmodulin activity (Stewart *et al.*, 1988). In *Ceratocystis ulmi*, addition of calcium can cause germination even in non-germination medium (Muthukumar & Nickerson, 1984); however, calcium had no such effect on *C. albicans*. Higher fungi appear not to have an absolute requirement of calcium for growth (Fletcher, 1982) hence it seems that a low internal concentration of calcium is sufficient for calcium-dependent events.

Germ tube formation is accompanied by an increase in the rate of ^{32}P incorporation. The inhibition of [^{32}P] incorporation both by Ca^{2+} depletion and by TFP suggests the involvement of Ca^{2+}-calmodulin-dependent protein kinases, which is the primary mechanism of calmodulin action in other systems. At least two germ-tube-specific and three bud-specific phosphoproteins can be identified (Fig. 4). Moreover, ^{31}P NMR spectroscopy studies have shown significant modulation in phosphorus-containing metabolites in the yeast-to-mycelial transition in *C. albicans* (Cassone *et al.*, 1983). The morphology-specific proteins reported elsewhere (Dabrowa & Howard, 1980; Finney *et al.*, 1985; Manning & Mitchell, 1980) appear to be different from those reported here.

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