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Calmodulin Levels in the Yeast and Mycelial Phases of *Ceratocystis ulmi*

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The calmodulin content of the yeast and mycelial phases of *Ceratocystis ulmi* was determined by radioimmunoassay. Calmodulin levels increased at the G1-S boundary of the cell cycle, coinciding with the first visible appearance of buds or germ tubes. However, in both phases the cellular calmodulin levels were equivalent. No differential synthesis was observed.

Calmodulin is a highly conserved protein, ca. 16,700 daltons in size, which contains four Ca(II) binding sites. It is thought to mediate many Ca(II) regulatory phenomena and to be ubiquitous in eucaryotic systems (5). Calmodulin has been detected in five filamentous fungi, *Agaricus bisporus* (4, 9), *Blastocladiella emersonii* (8), *Cortinarius* sp. (9), *Neurospora crassa* (18), and *Russula* sp. (9), but no distinctive function has yet been assigned to these fungal calmodulins. A function concerned with cell shape determination appears reasonable because Ca(II) has been shown to be a signal for hyphal branching in *N. crassa* (20), and Ca(II) gradients are thought to be essential for the growth of hyphal tips in *Achyla* sp. (19). In this light, we have been interested in the involvement of calmodulin in the regulation of yeast and mycelial dimorphism in the ascomycete *Ceratocystis ulmi* (Buism.) C. Moreau, the causative agent of Dutch elm disease. We have previously shown (16) that Ca(II) ions, the Ca(II) sequestering agent ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, the Ca(II) antagonist $LaCl_3$, and six calmodulin inhibitors (trifluoperazine, chlorpromazine, quinacrine, dibucaine, propranolol, and tetracaine) shifted the dimorphic potential of *C. ulmi*. These data were consistent with the conclusion (16) that a Ca(II)-calmodulin interaction was necessary for mycelial growth and that the absence of this interaction led to growth in the yeast phase. Of course, recognition of calmodulin involvement in fungal dimorphism does not explain the mechanism of that involvement. We now approach the question of calmodulin function in fungi by reporting the calmodulin levels present in the yeast and mycelial phases of *C. ulmi*. This study is the first such determination in a dimorphic fungus. Calmodulin has been detected in *Candida albicans* (11), but only the yeast phase was studied and no yields were reported.

MATERIALS AND METHODS

C. ulmi NRRL 6404 was used throughout this study, and blastospores and conidiospores were prepared as described previously (13). The defined glucose-salts media designated GPP and GPR contained L-proline and L-arginine, respectively, as the nitrogen source. Neither medium contained added calcium. *C. ulmi* exhibits a nutritional control of dimorphism in that it normally grows in the yeast phase in GPP and in the mycelial phase in GPR (13). Calmodulin levels were determined with a calmodulin-specific radioimmunoassay (RIA) kit (Amersham Corp., Arlington Heights, Ill.) by using sheep antibodies which had been produced in response to rat testis calmodulin and purified by affinity chromatography with a calmodulin-Sepharose 4B column. In the calmodulin-specific RIA kit, unlabeled rat calmodulin is used to construct the standard concentration curve. The specificity of the assay was confirmed by the absence of detectable calmodulin in homogenates of the bacteria *Agrobacterium tumefaciens* and *Bacillus thuringiensis* var. *israelensis*. At each time point the culture (50 ml) was harvested by centrifugation, washed, and resuspended in the Amersham calmodulin-extraction buffer supplemented with 5 mM EDTA and 0.6 mM phenylmethylsulfonyl fluoride. The cells were disrupted for 1 min at 4,000 rpm in a Braun MSK homogenizer with 0.3-mm glass beads. Cell breakage was 95 to 99% complete. All subsequent steps were as described in the RIA kit, with the calmodulin levels expressed as micrograms per milligrams of protein. Total protein in the supernatant was determined by the method of Bradford (1).

The protein, RNA, and DNA contents of the blastospores were determined as described by Nickerson et al. (17). Incorporation of radiolabeled adenine into alkali-stable trichloroacetic acid-precipitable material was by a modification of the method of Hartwell (10). Cells (4 ml of blastospores at 2×10^7 per ml) were pulsed for 15 min with 0.4 μCi of carrier-free [8-^{14}C]adenine (55.6 mCi/mmol). Duplicate 1-ml samples were added to tubes containing 1 ml of cold 10% trichloroacetic acid and stored overnight at 4°C. Then the samples were neutralized with 3 N NaOH, alkali hydrolyzed in 1 N NaOH at 25°C for 24 h, and reprecipitated with cold 30% trichloroacetic acid. The final concentration of trichloroacetic acid was 10 to 12%. This precipitate was collected on Whatman GFA filters and washed twice with 10 ml of cold 5% trichloroacetic acid containing nonradioactive adenine (10 μg/ml). The filters were transferred to scintillation vials, dried thoroughly, and counted.

RESULTS

Both blastospores (derived from yeasts) and conidiospores (derived from mycelia) contained a level of calmodulin equivalent to ca. 1% of the total buffer-soluble cell protein (Table 1). After inoculation the calmodulin content decreased three- to fourfold. These decreases may represent dilution via new protein synthesis (Table 2) or selective degradation of existing calmodulin. After inoculation the cellular protein and RNA content increased rapidly, whereas the DNA content remained virtually constant (Table 2). This pattern is typical of many fungal spores (17). The higher protein and RNA content of the GPR-grown cells (Table 2) is

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TABLE 1. Calmodulin levels in *C. ulmi*[a]

Time (h)	Calmodulin (μg/mg of protein) in:			
	Conidiospore inoculum		Blastospore inoculum	
	GPP	GPR	GPP	GPR
0	13.2 ± 1.2	13.2 ± 1.2	9.8 ± 1.8	9.8 ± 1.8
3	4.5 ± 1.1	5.2 ± 0.4	3.8 ± 1.9	4.0 ± 0.9
6	3.2 ± 0.1	3.8 ± 1.2	2.8 ± 1.1	2.7 ± 0.3
12	4.2 ± 0.4	2.9 ± 1.7	10.7 ± 0.6	8.3 ± 2.0
18	13.2 ± 2.3	10.3 ± 0.6	14.2 ± 1.1	12.0 ± 1.8

[a] The medium designated GPP contained proline as the nitrogen source and produced growth in the yeast phase, whereas GPR contained arginine and produced mycelia. Inocula were 2×10^7 spores per ml. Values are the means of three independent measurements ± standard errors.

due to the more rapid growth rate of *C. ulmi* with arginine as the nitrogen source (13). Pulse and continuous radiolabeling experiments with [U-^{14}C]phenylalanine and [2-^{14}C]cytosine confirmed that protein and RNA synthesis started immediately after blastospore inoculation and accelerated rapidly thereafter (R. K. Kulkarni, Ph.D. dissertation, University of Nebraska, Lincoln, 1981).

Cell calmodulin levels returned to their previous values between 12 and 18 h after inoculation (Table 1). The earlier increase observed with blastospore inocula probably reflects their more rapid development. By 18 h the spores were ca. 90% budded in GPP and 90% germinated in GPR (13; Fig. 1). This time interval was chosen so that the yeast phase growth had formed only a single bud and the mycelial growth had not yet formed any conidiospores. Significantly, with both spore types the cellular calmodulin levels were equivalent in the yeast (GPP) and mycelial (GPR) phases. There was no differential synthesis of calmodulin. However, the timing of the increased calmodulin levels (Table 1) corresponded with the first visible appearance of buds (Fig. 1a) or germ tubes (Fig. 1b) and the onset of DNA synthesis. In both GPP and GPR the incorporation of [8-^{14}C]adenine into alkali-stable trichloroacetic acid-precipitable material commenced 10 h after inoculation (Fig. 1). Thus, it appears that the *C. ulmi* spores are developmentally synchronized, and the calmodulin level increases at the G1-S boundary.

DISCUSSION

We have quantified the calmodulin level present in *C. ulmi* during the first 18 h of growth in the yeast and mycelial phases (Table 1). These data have been compared with other physiological (Table 2) and morphological (Fig. 1) events operative in the same time interval. Three conclusions can be drawn from our data.

(i) The RIA kit employing sheep antibody to rat testis calmodulin detects calmodulin from *C. ulmi*. Calmodulin is a highly conserved protein (5), and the RIA is known to be immunologically cross-reactive for calmodulins from vertebrate, invertebrate, and plant sources, as well as from *Chlamydomonas reinhardii* and *Dictyostelium discoideum* (3). Ours is the first study demonstrating that this cross-reactivity extends to filamentous or dimorphic fungi. The calmodulin levels we found in *C. ulmi* (Table 1) were substantially higher than the yields obtained from other filamentous fungi (9, 18). These differences may be real, resulting from the different species studied or from differences between dimorphic and nondimorphic fungi, or they may reflect differences in the techniques employed for the extraction, purification, and estimation of calmodulin. The RIA procedure frequently detects higher levels of calmodulin than alternative assays (3).

(ii) The level of cellular calmodulin is tied to the cell cycle with increases occurring at the G1-S boundary. The increase in calmodulin at 10 to 12 h coincides with the onset of DNA synthesis and the first visible appearance of buds or germ

TABLE 2. Macromolecular content of *C. ulmi* blastospores during development[a]

Time (h)	*C. ulmi* blastospore content (μg/mg, dry wt) in:					
	GPP			GPR		
	DNA	RNA	Protein	DNA	RNA	Protein
0	0.48	0.96	130	0.48	1.3	132
2	0.53	5.1	130	0.48	3.2	134
4	0.53	7.3	168	0.53	19.2	202
6	0.52	11.5	170	0.58	42.2	230
8	0.52	9.3	245	0.53	34.6	265
10	0.52	13.1	293	0.53	53.7	360
12	0.68	27.3	287	0.64	147	397

[a] GPP contained proline as the nitrogen source and produced growth in the yeast phase, whereas GPR contained arginine and produced mycelia. Lipid storage bodies constitute ca. 40% of the dry weight of *C. ulmi* blastospores (14).

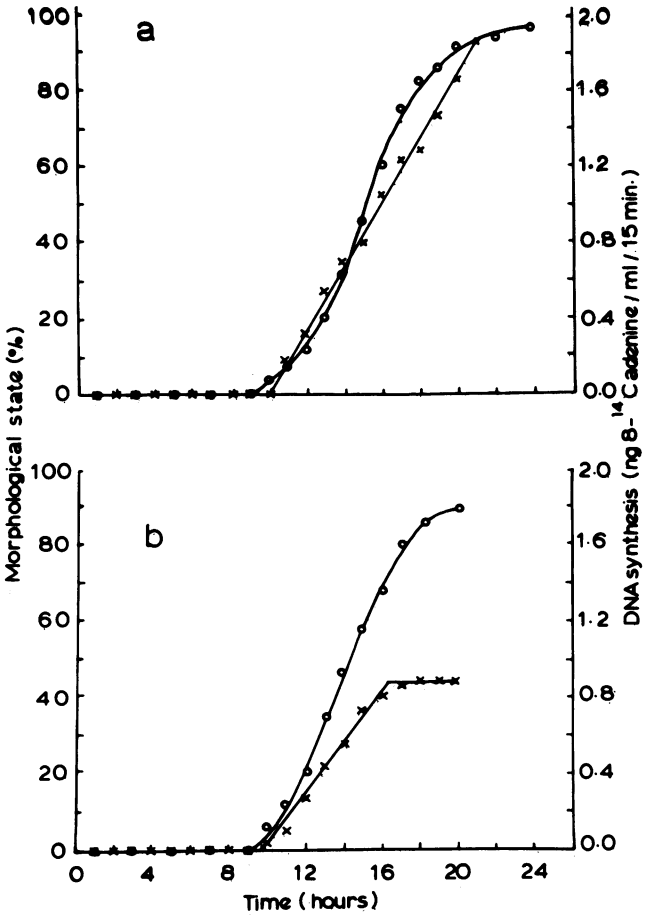

FIG. 1. Developmental morphology and DNA synthesis in *C. ulmi* blastospores. Panels: a, incubation in GPP; b, incubation in GPR. Symbols: X, DNA synthesis, 15-min pulse labeling with [8-^{14}C]adenine; O, percent budding in GPP (a) and percent germination in GPR (b).

tubes (Fig. 1). A similar cell cycle-dependent regulation of calmodulin was observed by Chafouleas et al. (2) in synchronized Chinese hamster ovary cells in which the calmodulin level doubled at the G1-S boundary (2).

(iii) Yeast and mycelia do not exhibit differential synthesis of calmodulin. With regard to cell shape, our previous data (16) indicated that a Ca(II)-calmodulin interaction was necessary for mycelial growth and the absence of this interaction led to growth in the yeast phase. One plausible model would have the calmodulin gene expressed only in the mycelial phase or to a significantly greater degree in the mycelial phase. Such a model would be consistent with the presence of calmodulin in filamentous fungi (4, 8, 9, 18); however, in three studies investigators using identical techniques were unable to detect calmodulin in *Saccharomyces cerevisiae* (7, 9, 12). Similarly, calmodulin levels differ in transformed and nontransformed cells. A two-to-threefold increase in calmodulin appears to be a general response to transforming agents in all of the cell systems studied so far (15). In the present study the differential synthesis model has been tested and disproved. In *C. ulmi* the cellular calmodulin levels are virtually identical in the yeast and mycelial phases (Table 1). These findings are in accord with the generalization of Cheung (6) that physiological functions of the Ca(II)-calmodulin complex are controlled by intracellular Ca(II) flux rather than by the cellular concentration of calmodulin.

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