Nutritional stress proteins in *Candida albicans*

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Starving cells of *Candida albicans* synthesize at least seven proteins that represent nutritional-stress proteins (NSP). Such NSPs are formed by both germination-competent and germination-deficient strains of *C. albicans*. Heat-shock proteins (HSP) are not formed by starving cells. Germination-competent cells synthesize specific sets of proteins when incubated in a starvation medium that contains the germ-tube-inducing substances *N*-acetyl-D-glucosamine or L-proline. Both sets of induced proteins were also synthesized by a germination-deficient strain of *C. albicans*.

Introduction

The blastoconidia of *Candida albicans* can initiate a hyphal form of growth by germination (Dabrowa & Howard, 1983). A search for germination-specific regulatory proteins has not been successful (Brown & Chaffin, 1981; Brummel & Soll, 1982; Finney *et al.*, 1985; and reviewed by Odds, 1988), but certain methods used to induce germ tube formation in *C. albicans* also evoke a stress response by the fungus (Dabrowa & Howard, 1984; Zeuthen *et al.*, 1988; Zeuthen & Howard, 1989). The stress response has been suggested to be involved in some aspects of growth and differentiation of cells (Carper *et al.*, 1987; LeJohn & Braithwaite, 1984; Lindquist, 1986; Schlesinger, 1986; Wanner *et al.*, 1985).

Cells of *Achlya klebsiana* grown in a nutritionally complete medium are induced to form asexual sporangia and to sporulate when they are transferred to a nutrient-free starvation medium (LeJohn & Braithwaite, 1984). The synthesis of 28 pre-existing proteins is markedly induced in cells transferred in this fashion. These nutritional-stress proteins (NSPs) are electrophoretically distinct from the heat-shock proteins (HSPs) induced in cells by a temperature shift from 24 °C to 37 °C. The two groups of proteins (NSP and HSP) were evidently induced independently. The development of asexual sporangia is arrested by the addition of glutamine to the

starvation medium. The supplement caused a reduction in the synthesis of NSPs but not of HSPs. These results led LeJohn & Braithwaite (1984) to speculate that NSPs but not HSPs played a role in reproductive development and sporulation. These studies on *A. klebsiana* lend some support to the notion that NSPs may be involved in morphogenesis.

We have been exploring the possible role of stress proteins formed by *C. albicans* in germination of its blastoconidia. Our approach has been to assess the occurrence of stress proteins in germination-competent and germination-deficient strains of the fungus (Howard *et al.*, 1986). We have described the heat-shock response (Dabrowa & Howard, 1984; Zeuthen & Howard, 1989) and the ethanol-stress response (Zeuthen *et al.*, 1988) in *C. albicans*. HSPs and the ethanol-stress proteins (ESPs) were expressed by both germination-competent and germination-deficient strains of *C. albicans*. In this report we present the results of our observations on the NSPs synthesized by starved cells of *C. albicans*.

Methods

Organisms. The *Candida albicans* strains used in the study were strain 300 (Dabrowa & Howard, 1984), strain 300-SG (Howard *et al.*, 1986) and strain 301 (B311-V6, Buckley *et al.*, 1982). Strain 300 formed germ tubes under standard test conditions (Zeuthen *et al.*, 1988) while strains 301 and 300-SG were germination-deficient (Buckley *et al.*, 1982; Howard *et al.*, 1986). Stock cultures of the strains are preserved in the Fungus Collection, University of California, Los Angeles, California, USA. Working cultures were maintained on GPA (see below) in a refrigerator and subcultured every month.

Media. Glucose/peptone agar (GPA) contained 2% (w/v) Bacto Dextrose (Difco), 1% (w/v) Bacto Peptone (Difco) and 2% (w/v) Bacto Agar (Difco). Glucose/peptone broth (GPB) had the same constituents

Abbreviations: ESP, ethanol-stress protein; GlcNAc, *N*-acetyl-D-glucosamine; GPA, glucose/peptone agar; HSP, heat-shock protein; LBC, synthetic medium of Lee *et al.* (1975); NSP, nutritional-stress protein; STM, starvation medium – 0.033 M-potassium phosphate buffer; YNB, yeast nitrogen base (without amino acids); YNBG, YNB + 1% (w/v) glucose.

without the agar. Yeast Nitrogen Base without amino acids (YNB) was prepared in accordance with the manufacturer's instructions (Difco). YNBG was YNB plus 1% (w/v) glucose. LBC synthetic medium was constructed as described by Lee *et al.* (1975). The starvation medium (STM) was 0.033 M-potassium phosphate buffer (pH 7.0). The effect of germ tube inducers on protein synthesis was studied in STM with 4 mM-*N*-acetyl-D-glucosamine (GlcNAc) and 2 mM-L-proline (Dabrowa *et al.*, 1976).

Studies on nutritional stress. Cells of strains 300 and 301 were grown for 16 h at 25 °C or 37 °C in YNBG. The cells were harvested, washed with distilled water, and resuspended in fresh YNBG or LBC and in STM at a concentration of 5×10^7 cells ml⁻¹. Three combinations of incubation temperatures were used in the preparation and study of cell populations: (i) cells grown at 25 °C and studied at 25 °C, (ii) cells grown at 37 °C and studied at 37 °C, and (iii) cells grown at 25 °C and studied at 37 °C. The cells were held in STM and complete medium (YNBG or LBC) for 30 min and 1 h. The response to nutritional stress was measured by radiolabelling proteins with L-[³⁵S]methionine (see below).

Studies on the heat-shock response. Cells of strains 300, 301 and 300-SG were grown for 16 h at 25 °C in YNBG, then harvested, washed in distilled water, and resuspended at 5×10^7 cells ml⁻¹ in YNBG prewarmed to 37 °C. The heat-shock period was 30 min at 37 °C. The heat-shock response was measured by radiolabelling proteins with L-[³⁵S]methionine (see below).

Studies on the response to germ tube inducers. Cells of strains 300, 301 and 300-SG were grown for 16 h at 25 °C in YNBG, then harvested, washed with distilled water, and resuspended in fresh YNBG or in STM with either 4 mM- or 2 mM-L-proline. The cells were incubated at 37 °C in the different sorts of media for 30 min and 1 h, and the response measured by radiolabelling proteins with L-[³⁵S]methionine (see below).

Germination. Cells of strain 300, 301 and 300-SG were grown for 16 h at 25 °C in YNBG, then harvested, washed in distilled water, and resuspended in LBC medium. The cultures were incubated at 37 °C and samples taken at various time increments. Germ tubes were observed after 1 h and were present on 80–90% of the cells in 3 h (Zeuthen *et al.*, 1988).

Radiolabelling techniques. Proteins were labelled by adding L-[³⁵S]methionine (specific activity 45 TBq mmol⁻¹) to the cultures at a final concentration of 3.7 MBq ml⁻¹ for the periods 0–30 min or 30–60 min after transfer to new media. The HSPs were labelled for the period 0–30 min after temperature shift. Labelling was stopped by addition of unlabelled DL-methionine (final concentration 650 µM). The cells were immediately placed on ice, washed in distilled water, and held as a pellet at –20 °C until they were prepared for electrophoresis.

Polyacrylamide gel electrophoresis (PAGE). The frozen cells from experiments designed to study the stress response were thawed and suspended in 0.4 ml 1 mM-phenylmethylsulphonyl fluoride in water. Glass beads (2 vols, 0.45 mm) were added to each tube. Cells were broken by mixing on a vortex mixer (Vortex-Genie, Scientific Products) at maximum speed for six periods of 30 s each. Cells were kept on ice except during vortex mixing. Microscopic examination revealed that 90–95% of the cells were broken. Beads and cell wall debris were pelleted by centrifugation. The supernatant containing crude proteins was added to an equal volume of 0.1 M-Tris/HCl (pH 6.8) containing 18% (v/v) glycerol, 1.8% (w/v) SDS, 0.18% 2-mercaptoethanol and 0.0018% bromophenol blue, and was immediately heated at 100 °C for 2 min. The radiolabelled proteins in the cell extracts were separated by one-dimensional SDS-PAGE in 12% polyacrylamide slab gels (Dabrowa & Howard, 1984). The time of electrophoresis was approximately 3 h. The amount of protein per lane

was 10–15 µg. Proteins were separated at a constant current of 25 mA, fixed in methanol/acetic acid/water (5:1:4, by vol.), and stained by the silver nitrate method (Morrissey, 1981). After staining, the gels were dried onto GelBond PAG support paper (FMC BioProducts, Rockland, Maine, USA) and exposed to Kodak XAR-5 film at –70 °C. Molecular mass markers were from Pharmacia. Reagents for electrophoresis were obtained from BRL and Sigma.

Results and Discussion

Nutritional stress proteins synthesized by C. albicans

The pattern of protein synthesis by cells of *C. albicans* strain 300 grown at 25 °C in YNBG and transferred to fresh YNBG or to STM is shown in Fig. 1. Protein synthesis was not obviously suppressed in cells suspended in STM. A few of the protein bands observed in extracts from cells incubated in YNBG (Fig. 1, lane 1) were reduced in intensity in the extracts from cells suspended in STM (Fig. 1, lane 2). This result is in contrast to that observed in ethanol stress, where exposure to ethanol shuts off nearly all protein synthesis except that of the ESPs (Zeuthen *et al.*, 1988). Nine proteins (73, 71, 64, 55, 45, 42, 41, 38 and 34 kDa) were synthesized either uniquely or at markedly enhanced levels by cells in STM and could be considered NSPs. The results shown in Fig. 1 were obtained with cells starved for 30 min. It is known that starvation for as little as 20 min will prepare cells of *C. albicans* for germination (Soll *et al.*, 1985). Cells starved for 1 h and pulse-labelled during the period 30–60 min provided the same results as those shown in Fig. 1 (data not presented). Cells with germ tubes were observed after 1 h in LBC medium at 37 °C, a time period in keeping with the observations of others (Sevilla & Odds, 1986). These same results were obtained with cells from three combinations of incubation temperatures used in the preparation and study of cell populations: (i) cells grown at 25 °C and studied at 25 °C (Fig. 1), (ii) cells grown at 37 °C and studied at 37 °C (data not shown), and (iii) cells grown at 25 °C and studied at 37 °C (data not shown). Specific proteins synthesized in response to the temperature shift from 25 °C to 37 °C (HSPs) were not detected in cells suspended in STM. Thus, even though some of the NSPs are electrophoretically synonymous with HSPs (Zeuthen & Howard, 1989) the two sets of proteins are distinctively evoked.

Comparison of NSPs formed by germination-competent and germination-deficient strains of C. albicans

Starvation has been reported to foster germ tube formation by yeast cells of *C. albicans* (Shepherd *et al.*, 1980; Soll *et al.*, 1985). The notion that NSPs formed in

Fig. 1

Fig. 2

Fig. 1. Autoradiogram of cellular proteins separated by one-dimensional SDS-PAGE (12%, w/v, polyacrylamide). Cells of *C. albicans* strain 300 were grown for 16 h in YNBG at 25 °C and then transferred to fresh YNBG or to STM. The cells were labelled with [35 S]methionine during the 0–30 min period after the transfer to the two sorts of media. Lane 1, labelled proteins extracted from cells incubated in YNBG; lane 2, labelled proteins extracted from cells incubated in STM. Location of molecular mass markers is indicated on the left of the figure. NSPs are indicated on the right of the figure. The data are from a representative experiment repeated twice. Only those NSPs that were consistently noted in all runs are indicated.

Fig. 2. Autoradiogram of cellular proteins separated by one-dimensional SDS-PAGE (12%, w/v, polyacrylamide). Cells of *C. albicans* strain 300 (germination-competent) and strain 301 (germination-deficient) were grown for 16 h in YNBG at 25 °C and then transferred to LBC or STM. The cells were labelled with [35 S]methionine during the 0–30 min period after the transfer to the two sorts of media. Lanes 1, 2 and 3 are labelled proteins from strain 300; lanes 4, 5 and 6 are labelled proteins from strain 301. Lanes 1 and 4, STM at 25 °C; lanes 2 and 5, STM at 37 °C; lanes 3 and 6, LBC at 37 °C. Location of molecular mass markers is indicated on the left of the figure. NSPs are indicated on lane 4. The data are from a representative experiment repeated three times. Two of the repetitions were conducted with strain 300-SG.

response to starvation might play a role in germ tube formation would be strengthened if the occurrence of such proteins were different in germination-competent and germination-deficient strains of *C. albicans*. The proteins synthesized by cells of strain 300 (germination-competent) and strain 301 (germination-deficient) grown in YNBG at 25 °C for 16 h and then transferred to LBC or to STM are shown in Fig. 2. The results shown are from a pulse label during the 0–30 min increment, but the same bands were observed in materials from cells during the 30–60 min increment (data not shown). Cells of *C. albicans* are prepared for germination after starvation for 20 min in a nutrient-free medium (Soll *et al.*, 1985). LBC was used as the complete medium in this comparative study because yeast cells germinate when incubated in it at 37 °C for 1 h (data not shown). Yeast cells do not germinate in YNBG at 37 °C (Dabrowa & Howard, 1983).

The use of LBC instead of YNBG as the complete medium led to slightly different assessment of the NSPs. A few of the bands were not synthesized or were only weakly manifested by cells in LBC but were synthesized by cells in YNBG. These nutritionally modulated proteins probably should not be considered NSPs, since their appearance as such depends on the complete medium used in comparisons. From these considerations the NSPs observed under the conditions employed in our study are 73, 71, 64, 55, 45, 38 and 34 kDa. In spite of these difficulties, it is clear that the seven unequivocal NSPs were formed by both germination-competent and germination-deficient strains (Fig. 2). Thus, there is no evidence from these experiments that the NSPs observed were involved in germ tube formation.

Protein synthesis in heat-shocked growing and starving cells of C. albicans

There is abundant evidence that HSPs can be induced by other stresses such as ethanol (Zeuthen *et al.*, 1988), heavy metals (Zeuthen & Howard, 1989), and starvation (LeJohn & Braithwaite, 1984). Nevertheless, in some fungi the HSPs and NSPs appear to be under independent control (LeJohn & Braithwaite, 1984). The results shown in Fig. 3 indicate that three prominent HSPs can be identified that are formed in complete medium but are not seen in extracts from cells incubated in STM or STM with GlcNAc. These same results were obtained with the germination-deficient strains 301 and 300-SG. These results reaffirm the fact that HSPs are not synthesized by cells of *C. albicans* suspended in STM and subjected to a temperature shift (25 °C to 37 °C) that will evoke them by cells in complete medium. Cells of *A. klebsiana* synthesized HSPs in a starvation medium (LeJohn & Braithwaite, 1984). However, the medium and the

Fig. 3. Autoradiogram of cellular proteins separated by one-dimensional SDS-PAGE (12%, w/v, polyacrylamide). Cells of *C. albicans* strain 300 were grown in YNBG for 16 h at 25 °C and then transferred to fresh YNBG (lane 1), to LBC (lane 2), to STM (lane 3), and to STM with 4 mM-GlcNAc (lane 4), at 37 °C. The cells were labelled with [³⁵S]methionine during the 0–30 min period after the transfer to the different sorts of media. Three prominent HSPs are indicated by arrows. The data are from a representative experiment repeated three times.

conditions were different from those that we employed with *C. albicans*.

Modulation of protein synthesis by inducers of germ tube formation

Blastoconidia of *C. albicans* that are suspended in STM will germinate when supplied with inducers such as GlcNAc or proline (Dabrowa & Howard, 1983; Gopal *et al.*, 1982; Shepherd *et al.*, 1980; Simonetti *et al.*, 1974). The induced cells synthesize specific sets of proteins that are not formed by cells incubated in STM alone (Dabrowa & Howard, 1988; Torosantucci *et al.*, 1984; Angiolella *et al.*, 1986). The induced proteins of particular interest would be those formed in the presence of an inducer by a germination-competent strain of *C. albicans* (300) but not by a germination-deficient strain

(301). Several experiments were conducted to search for such germination-specific proteins but none was detected. Thus, there is no evidence from our work with germination-competent and germination-deficient strains that the proteins synthesized in a starvation medium supplemented with strong germ tube inducers play a role in regulation of germination.

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References

- ANGIOLELLA, L., TOROSANTUCCI, A., CARRUBA, G. & CASSONE, A. (1986). Nutrition-dependent modulations of protein synthesis in *Candida albicans* during germ-tube formation and maintenance of the yeast form in *N*-acetylglucosamine media. *FEMS Microbiology Letters* **36**, 231–237.
- BROWN, L. A. & CHAFFIN, W. L. (1981). Differential expression of cytoplasmic proteins during yeast bud and germ tube formation in *Candida albicans*. *Canadian Journal of Microbiology* **27**, 580–586.
- BRUMMEL, M. & SOLL, D. R. (1982). The temporal regulation of protein synthesis during synchronous bud or mycelium formation in the dimorphic yeast *Candida albicans*. *Developmental Biology* **89**, 211–224.
- BUCKLEY, H. R., PRICE, M. R. & DANEOMOORE, L. (1982). Isolation of a variant of *Candida albicans*. *Infection and Immunity* **37**, 1209–1217.
- CARPER, S. W., DUFFY, J. J. & GERNER, E. W. (1987). Heat shock proteins in thermotolerance and other cellular processes. *Cancer Research* **47**, 5249–5255.
- DABROWA, N. & HOWARD, D. H. (1983). Blastocnidium germination. In *Fungi Pathogenic for Humans and Animals*, Part A, *Biology*, pp. 525–545. Edited by D. H. Howard. New York: Marcel Dekker.
- DABROWA, N. & HOWARD, D. H. (1984). Heat shock and heat stroke proteins observed during germination of the blastocnidia of *Candida albicans*. *Infection and Immunity* **44**, 537–539.
- DABROWA, N. & HOWARD, D. H. (1988). Nutritionally induced changes in protein synthesis of *Candida albicans*. *Abstracts of the Annual Meeting of the American Society for Microbiology*, 1988, F2, p. 392.
- DABROWA, N., TAXER, S. S. S. & HOWARD, D. H. (1976). Germination of *Candida albicans* induced by proline. *Infection and Immunity* **13**, 830–835.
- FINNEY, R., LANGTIMM, C. J. & SOLL, D. R. (1985). The programs of protein synthesis accompanying the establishment of alternative phenotypes in *Candida albicans*. *Mycopathologia* **91**, 3–15.
- GOPAL, P., SULLIVAN, P. A. & SHEPHERD, M. G. (1982). Enzymes of *N*-acetylglucosamine metabolism during germ-tube formation in *Candida albicans*. *Journal of General Microbiology* **128**, 2319–2326.
- HOWARD, D. H., ZEUTHEN, M. L. & DABROWA, N. (1986). Phenotypic characteristics of a slow-growing, nongerminative variant of *Candida albicans*. *Journal of General Microbiology* **132**, 2359–2366.
- LEE, K. L., BUCKLEY, H. R. & CAMPBELL, C. C. (1975). An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**, 148–153.
- LEJOHN, H. B. & BRAITHWAITE, C. E. (1984). Heat and nutritional shock-induced proteins of the fungus *Achlya* are different and under independent transcriptional control. *Canadian Journal of Biochemistry and Cell Biology* **62**, 837–846.
- LINDQUIST, S. (1986). The heat-shock response. *Annual Review of Biochemistry* **55**, 1151–1191.
- MORRISSEY, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* **117**, 307–310.
- ODDS, F. C. (1988). *Candida and Candidosis*, 2nd edn. London: Baillière Tindall.
- SCHLESINGER, M. J. (1986). Heat shock proteins: the search for functions. *Journal of Cell Biology* **103**, 321–325.
- SEVILLA, M.-J. & ODDS, F. C. (1986). Development of *Candida albicans* hyphae in different media – variation in growth rates, cell dimensions and timing of morphogenetic events. *Journal of General Microbiology* **132**, 3083–3088.
- SHEPHERD, M. G., YIN, C. Y., RAM, S. P. & SULLIVAN, P. A. (1980). Germ tube induction in *Candida albicans*. *Canadian Journal of Microbiology* **26**, 21–26.
- SIMONETTI, N., STRIPPOLI, V. & CASSONE, A. (1974). Yeast-mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. *Nature, London* **250**, 344–346.
- SOLL, D. R., HERMAN, M. A. & STAEHELL, M. A. (1985). The involvement of cell wall expansion in the two modes of mycelium formation of *Candida albicans*. *Journal of General Microbiology* **131**, 2367–2375.
- TOROSANTUCCI, A., ANGIOLELLA, L., FILESI, C. & CASSONE, A. (1984). Protein synthesis and amino acid pools during yeast-mycelial transition induced by *N*-acetyl-D-glucosamine in *Candida albicans*. *Journal of General Microbiology* **130**, 3285–3293.
- WANNER, R., FORSTER, H., MENDGEN, K. & STAPLES, R. C. (1985). Synthesis of differentiation-specific proteins in germlings of the wheat stem rust fungus after heat shock. *Experimental Mycology* **9**, 279–283.
- ZEUTHEN, M. L. & HOWARD, D. H. (1989). Thermotolerance and the heat-shock response in *Candida albicans*. *Journal of General Microbiology* **135**, 2509–2518.
- ZEUTHEN, M. L., DABROWA, N., ANIEBO, C. M. & HOWARD, D. H. (1988). Ethanol tolerance and the induction of stress proteins by ethanol in *Candida albicans*. *Journal of General Microbiology* **134**, 1375–1384.