Synthesis of heat-shock proteins in mycelia and yeast forms of *Paracoccidioides brasiliensis*

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Summary. The induction of heat-shock proteins has been postulated to play a role not only in thermo-adaptation, but also in phase transition of the dimorphic fungi. In this study, we used yeast and mycelial forms of the thermally dimorphic fungus *Paracoccidioides brasiliensis* to evaluate the effect of temperature on the induction of the heat-shock response. We also evaluated protein synthesis by *P. brasiliensis* caused by exposure to low pH and H₂O₂. Analysis of protein synthesis by SDS-PAGE disclosed that *P. brasiliensis* mycelia increased synthesis of all major constitutive proteins when stressed at 37°C and increased synthesis of three non-constitutive proteins of 134, 82 and 28 kDa at 40°C. Yeasts incubated at 40°C showed decreased synthesis of five constitutive proteins (136, 98, 62, 57 and 54 kDa) and the appearance of three new proteins (134, 82 and 28 kDa). There was a decrease in the synthesis of all major constitutive proteins except for three proteins of 141, 136 and 16 kDa when yeast cells were incubated at 25°C. When stressed by low pH and H₂O₂, *P. brasiliensis* yeast increased synthesis of one (134 kDa) and five (134, 104, 82, 52 and 40 kDa) non-constitutive proteins, respectively. *P. brasiliensis* mycelia and yeast forms disclosed the same profile of protein synthesis when stressed at temperatures that trigger phase transition (37°C for mycelia; 25°C for yeast). The same profile of protein synthesis by both forms occurred when the fungi were incubated at 40°C and was similar to that of yeast cells stressed by low pH or H₂O₂, but different from the patterns produced by mycelia incubated at 37°C or yeast at 25°C. These results suggest that synthesis of stress proteins by *P. brasiliensis* mycelia and yeast forms at 40°C, low pH or exposed to H₂O₂ was associated with adaptation to hostile environments. In contrast, the overall increased and decreased synthesis of major constitutive proteins by mycelia and yeast forms at 37°C and 25°C was associated with phase transition. It is unlikely that the heat-shock proteins produced in these experiments are important in the maintenance of the morphology of yeast or mycelia at their usual temperatures of growth.

Introduction

Paracoccidioides brasiliensis, a thermally dimorphic fungus, is the aetiological agent of paracoccidioidomycosis, the most prevalent systemic mycosis in Latin America.¹ The fungus grows as a multicellular mycelium at 25°C but as a unicellular yeast at 37°C.² Temperature shifts between 25°C and 37°C trigger the transition from one form to the other. The mycelial to yeast-phase transition is of particular interest, as conversion to the yeast morphology is thought to be important in the pathogenicity of dimorphic fungi. Virtually all micro-organisms synthesise increased amounts of certain proteins when exposed to heat and other forms of stress, and these proteins might be important factors in the mechanism of resistance to an adverse environment.^{3,4} However, in dimorphic organisms, such as *P. brasiliensis*, heat-shock proteins may

conceivably play an important role in the phase-transition process.^{5,6}

In the present study, we evaluated the induction of stress proteins in both mycelia and yeast forms of *P. brasiliensis* at different temperatures and other conditions of stress (low pH and H₂O₂). The two non-thermal stresses were selected as an initial survey of conditions potentially present during fungal interactions with host defence cells, specifically phagocytes. The results of this work suggest that the heat-shock response is an adaptation to external stresses and not necessarily an important factor in the transition of one growth form to the other. Moreover, the responses to thermal and non-thermal stresses are different.

Materials and methods

Organism

P. brasiliensis strain DY, a virulent isolate from a patient with paracoccidioidomycosis, was kindly pro-

vided by R. Martinez, Ribeirão Preto School of Medicine, University of São Paulo and was passaged in our laboratory. The fungus was identified as *P. brasiliensis* by the presence of the characteristic "pilot wheel" morphology of the yeast forms at 37°C and the ability of yeast to complete transition to the mycelial form at 25°C. Yeast and mycelial cells were grown to the mid-log phase (48 h for yeast and 6–10 days for mycelia) in Brain Heart Infusion Broth (Difco) at 37°C and 25°C, respectively. Growth was interrupted, the fungi were washed in phosphate-buffered saline (PBS) and were resuspended at a concentration of 5×10^7 yeast cells or 3 ml/g wet weight of mycelia in PBS pre-warmed to the different temperatures studied (25°C, 37°C and 40°C).

Protein labelling

Fungi were incubated for 15 min at the desired conditions before the addition of the radioactive-labelled methionine. Proteins were then labelled with L-[^{35}S]methionine (specific activity 45 TBq/mmol) to a final concentration of 3.7 MBq/ml for 30 min in all of the heat-shock and stress response experiments. In the pH exposure experiments, *P. brasiliensis* yeasts were resuspended in PBS and adjusted to pH 4.0 with 0.6 M HCl at 37°C. In the H_2O_2 experiments, *P. brasiliensis* was incubated in 3 mM H_2O_2 at 37°C. Radioactive incorporation was stopped by the addition of unlabelled DL-methionine (final concentration 650 μM). Experiments were performed three times, with similar results in each experiment.

SDS-PAGE

After termination of the test incubation, the cells were immediately placed on ice, washed in PBS and suspended in 0.4 ml of 1 mM phenylmethylsulphonyl fluoride in water. Sterile glass beads (2 vols, 0.45 mm) were added to each tube. Cells were broken by mixing on a vortex mixer (Vortex-Genie) at maximum speed for 10 periods of 1 min each. Additionally, mycelial cells were broken with a mortar and pestle as described by Akins and Lambowitz.⁷ Beads and broken cell walls were pelleted by centrifugation (2000 *g* for 10 min). The supernate was collected and precipitated with trichloroacetic acid 10% at 0°C for 15 min. The precipitate was centrifuged at 9000 *g* for 5 min, washed with acetone and dried under a vacuum. The protein pellet was added to 100 μl of 0.1 M Tris-HCl (pH 6.8) containing glycerol 18% v/v, SDS 1.8% w/v, 2-mercaptoethanol 0.18% and bromophenol blue 0.0018%, and was immediately heated at 100°C for 5 min. Each lane contained the protein recovered from 5×10^7 yeasts or 3 ml/g wet weight of mycelia. In one experiment, the volume of the samples was adjusted to provide equal numbers of cpm/lane. The results of this experiment were similar to those obtained by loading the same numbers of yeast or equivalent weights of mycelia to each lane. The radiolabelled proteins were

separated by one-dimensional SDS gradient gel electrophoresis on polyacrylamide 4–15% or 4–20% w/v slab gels. Molecular mass standards—phosphorylase b (106 kDa); bovine serum albumin (80 kDa); ovalbumin (49 kDa); carbonic anhydrase (32 kDa); soybean trypsin inhibitor (27 kDa); and lysozyme (18 kDa)—used to estimate the molecular mass of proteins separated by SDS-PAGE were purchased from Sigma. Gels were dried and exposed to Kodak XAR-5 film at -70°C . Film was developed after exposure for 72 h.

Results

Effect of changing incubation temperature on yeast cells

As shown in fig. 1, yeast cells, harvested during exponential growth phase and incubated with ^{35}S -methionine at 37°C, synthesised *c.* 12 proteins with

Fig. 1. Autoradiogram of cellular protein synthesis by *P. brasiliensis* yeast (DY) determined by SDS-PAGE (4–15% gradient gel) analysis of ^{35}S -methionine-labelled proteins. Lane 1, 25°C; 2, 37°C; 3, 40°C. Note the decreased synthesis of all major proteins at 25°C (–) and the synthesis of three HSPs (+) at 40°C. Molecular mass markers (kDa) are indicated on the left. ← constitutive 40-kDa protein.

Table. Profile of protein synthesis by mycelia and yeast forms of *Paracoccidioides brasiliensis* incubated at different temperatures

Growth form	Profile changes at incubation temperature of								
	25°C			37°C			40°C		
	Decrease	No change	Increase	Decrease	No change	Increase	Decrease	No change	Increase
Mycelia	...	Baseline constitutive synthesis	...	...	...	Global increase in constitutive proteins	136, 96, 62, 57, 54 kDa	...	134, 82 and 28 kDa new bands
Yeast	Global decrease in constitutive protein synthesis	...	No new bands	...	Baseline constitutive protein synthesis	...	136, 96, 62, 57, 54 kDa	...	134, 82 and 28 kDa new bands

All band masses represent approximate molecular mass.

Fig. 2. Autoradiogram of cellular protein synthesis by *P. brasiliensis* mycelia (DY) determined by SDS-PAGE (4–15% gradient gel) analysis of ^{35}S -methionine-labelled proteins. Lane 1, 25°C; 2, 37°C; 3, 40°C. Note the increased synthesis of all major proteins at 37°C (+). Synthesis of three HSPs (+) was observed at 40°C when compared to 37°C. Molecular mass markers (kDa) are indicated on the left; ← constitutive 40-kDa protein.

Fig. 3. Autoradiogram of cellular protein synthesis by *P. brasiliensis* yeast (DY) determined by SDS-PAGE (4–20% gradient gel) analysis of ^{35}S -methionine-labelled proteins. Lane 1, cells labelled at 37°C not exposed to stress; 2, cells exposed to low pH; 3, cells exposed to H_2O_2 . Note the synthesis of non-constitutive proteins when yeast cells were stressed by low pH or H_2O_2 . Molecular mass markers (kDa) are indicated on the left; ←, constitutive 40-kDa protein.

apparent molecular masses ranging from 140 to 15 kDa. *P. brasiliensis* yeast cells, when incubated at 40°C, produced at least three non-constitutive proteins (134, 82 and 28 kDa) and decreased synthesis of five constitutive proteins (136, 98, 62, 57 and 54 kDa;

fig. 1; table). In contrast, there was a decrease in the synthesis of all major constitutive proteins when yeasts were incubated at 25°C except for the persistence of three proteins of 141, 136 and 14 kDa (fig. 1).

Effect of changing incubation temperature on mycelical cells

The constitutive protein synthesis of exponential growth phase mycelial cells is shown in fig. 2. There was an overall increase in all major protein bands when mycelia were incubated at 37°C, even though there were no new bands identifiable on the autoradiograms. However, when the incubation temperature was raised to 40°C, three new proteins appeared that had apparent molecular masses similar to those seen in yeast cells incubated at this higher temperature (134, 82 and 28 kDa; table).

Effect of acid pH on yeast cells

When incubated at pH 4.0, most constitutive protein synthesis present during usual culture conditions demonstrated no change. One new protein band appeared (134 kDa) and three bands with apparent molecular masses of 136, 108 and 104 kDa decreased (fig. 3).

Effect of H₂O₂ on yeast cells

As shown in fig. 3, incubation of *P. brasiliensis* yeast cells with 3 mM H₂O₂ induced the synthesis of five new protein bands (c. 134, 104, 82, 52 and 40 kDa). A decrease in three protein bands (136, 110 and 108 kDa) observed under the low pH stress conditions also occurred (fig. 3).

Discussion

P. brasiliensis mycelia and yeast forms respond to exposure to supra-optimal temperatures with an obviously altered pattern of protein synthesis (summarised in the table). Similar responses have been observed with other organisms. New bands of c. 134, 82 and 28 kDa were observed in both yeast and mycelia that were incubated at 40°C and these responses were similar. Interestingly, new protein bands were not observed when the incubation temperature of mycelia was increased to 37°C, a temperature often used to induce transition to the yeast form. Thus, the production of heat-shock proteins, under the conditions used in these experiments, does not appear to be necessary for the initiation or maintenance of phase transition.

There are some similarities between the new stress proteins identified in this study and those observed by others in different organisms. For example, heat-shock proteins of 82 and 28 kDa are similar in molecular size to the conserved families of HSPs at 80 and 30 kDa, respectively.⁸ The *P. brasiliensis*-derived heat-shock protein of c. 134 kDa is similar to the high molecular mass stress protein recorded in *Candida albicans* cells stressed with CdCl₂.⁹ However, the typical heat-shock response of the production of novel proteins accom-

panied by a marked decrease in constitutive protein synthesis, as seen in *C. albicans* and *Neurospora crassa*,^{9,10} was not seen in mycelia "stressed" by incubation at 37°C. Kamei *et al.* described increased synthesis of six constitutive proteins and decreased synthesis of six constitutive proteins by *Histoplasma capsulatum* yeasts incubated at 40°C.¹¹ This is similar to the results obtained in the present study.

Matthews has described a 47-kDa antigen in *C. albicans*, which is recognised by sera of patients with invasive candidiasis.¹² This 47-kDa antigen is a component of HSP 90. Thus, there is a similarity in host response to both *C. albicans* and *P. brasiliensis* in that a potentially diagnostically useful antigen is synthesised by the fungi as a heat-shock protein.

The induction of heat-shock proteins has been postulated to play a role not only in thermo-adaptation but also in cell differentiation.^{2,5,6} Dimorphic fungi, such as *P. brasiliensis*, may be particularly useful models for the study of heat-shock proteins, as the development of the two morphological forms is regulated in nature by changes in ambient temperature,¹³ yet the organism responds to both thermal and non-thermal stresses with changes in protein synthesis typical of the evolutionarily conserved heat-shock response. In the search for the molecular events responsible for phase transition, it is important to distinguish between the effect of temperature on morphogenesis and on the heat-shock response. The bands present on autoradiograms of mycelia incubated at 37°C for the relatively short incubation period of 45 min were similar to those of the constitutive protein synthesis of yeasts maintained at that temperature. However, Clemons *et al.* showed that mycelia and yeasts demonstrated different patterns of protein synthesis after much longer incubation periods than were studied in these experiments.¹⁴

Mycelia and yeast forms incubated at 40°C demonstrated similar responses to this thermal stress and these responses were similar to those seen when yeasts were exposed to either acid pH or the oxidant H₂O₂. Previous studies have shown that proteins synthesised in response to different forms of stress may be determined by the nature of the stress.¹⁵ It is also known that some proteins synthesised in response to one stimulant can be evoked by other stresses. It is notable that the heat-shock responses of mycelia and yeast incubated at 40°C, and yeast exposed to low pH or H₂O₂, are similar. This suggests that the reaction to diverse "stresses" may be an adaptive response of the fungus and that the new proteins that appear are unlikely to be associated in a fundamental way with phase transition.

Both incubation in an acid environment (pH 4.0) or with H₂O₂ (3 mM) induced the synthesis of one or five new proteins, respectively, and a decrease in the synthesis of three constitutive proteins. It is of note that new proteins produced by thermal stress were distributed over broad apparent molecular masses (136–28 kDa), but most of the proteins synthesised in

response to the other two stresses were > 80 kDa. It is not known whether the lower molecular bands seen in the fungi exposed to high temperatures represent degradation or processing of the higher molecular mass proteins. Alternatively, these stress proteins might represent an entirely unique response to non-thermal stress. The role of these proteins and the significance of the disappearance of specific proteins after exposure to acid pH or H₂O₂ would be of interest as these represent conditions present during the confrontation between micro-organisms and phagocytes.

The increase in synthesis of proteins in the 40–43 kDa range, constitutively expressed by yeasts incubated at 37°C or mycelia exposed to 37°C, but decreased in yeast exposed to 25°C, is interesting as an antigen in this size range has been found to be highly immunogenic and antibodies to a protein in this molecular mass range have been identified in sera from patients with paracoccidioidomycosis.^{16,17} Moreover,

we have shown in these experiments that these proteins are synthesised when yeast are exposed to acid pH or H₂O₂, conditions likely to be encountered by yeasts invading a mammalian host. Whether the higher molecular mass proteins that were found to be increased in this study are related to this antigen remains to be determined.

In summary, we have demonstrated that the heat-shock response of the dimorphic fungus, *P. brasiliensis* occurs at 40°C in both mycelia and yeasts and that this response is similar to that seen in yeast cells stressed by incubation at a low pH or in the presence of H₂O₂. Future studies should focus on the role of these stress proteins in the protection of the fungus from both the cellular and humoral immune response of an infected host.

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