CURRENT MICROBIOLOGY Vol. 26 (1993), pp. 123–127

Current Microbiology

Multiple Responses to Heat Stress by the Basidiomycete *Schizophyllum commune*

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Abstract. Temperatures in excess of 45°C are required to stop the growth of *Schizophyllum commune* colonies. Transfer of colonies from normal growth conditions (21°C) to 55°C, while halting mycelial expansion and increasing the production of aerial hyphae, was not lethal. Short-term heat shock (3 h) resulted in the appearance of nine proteins resolvable by SDS–PAGE that were newly synthesized or had their synthesis increased. The molecular weights of these proteins qualify two of them as being members of the hsp90 and hsp70 families of heat shock proteins. Heat shock also affected proteolytic processes in the colonies. Changes in the pattern of ubiquitinated protein conjugates occurred; fewer high-molecular-weight conjugates were found in heat-shocked colonies, and the appearance of a ladder of lower-molecular-weight conjugates was noted. Protease enzymes detected by gelatin-gel PAGE showed a general decrease in activity. One of these proteases, which was up-regulated during nitrogen deprivation, showed an intermediate response during the combined stresses of heat shock and nitrogen starvation.

Virtually all organisms, upon a shift to higher than normal growth temperatures, have been shown to exhibit a common array of cellular responses. The most striking of these responses is the increased production of a group of proteins referred to as heat shock proteins (hsps). The regulation of the heat shock response and the transcription of heat shock proteins have received in-depth investigation in a few microorganisms, especially *Saccharomyces cerevisiae* and *Escherichia coli* [12, 13, 16]. Study of heat shock in filamentous fungi has been limited principally to *Neurospora crassa* [18], *Aspergillus nidulans* [22], and *Achlya ambisexualis* [21]. Within the basidiomycetes, the heat shock response has been documented only in the plant pathogen *Ustilago maydis* [8].

Schizophyllum commune is a widely distributed basidiomycete, growing primarily on dead and decaying wood. As such, it is likely to be exposed to numerous environmental stresses including high temperatures and nutrient deprivation. Of particular interest to us is the role of proteolysis in these stress responses. We have reported previously that nitrogen deprivation leads to increased general proteolysis and a change in the spectrum of proteases detectable in gelatin-containing polyacrylamide gels [10, 11]. An integral part of the heat-shock response also involves proteolysis. The best characterized heat shock-related protein degradation occurs by ubiquitin-mediated proteolysis [5, 7]; however, it is clear that other systems operate also. The peptide sequence KFERQ and related sequences serve as signal sequences that target intracellular proteins for lysosomal degradation [4]. A 73-kilodalton (kDa) intracellular protein from rat liver, established as a member of the hsp70 family, binds the KFERQ sequence and translocates proteins containing the sequence into a lysosome [3, 23]. This directly links the heat-shock response to proteolytic channels other than the ubiquitin-mediated ones.

Here we report several responses of *S. commune* to heat shock, including the production of hsps, ubiquitination of proteins, and changes in proteases.

Materials and Methods

Fungal culturing and growth. The *S. commune* stock culture used in this study, homokaryon 4-39 (*A41/B41*), is descended from the original Raper 699 strain. Stock cultures were maintained on the

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surface of agar-containing minimal medium plates that had been covered with a cellophane membrane [1]. A 3 × 3 mm square of the cellophane membrane supporting the mycelium of the advancing hyphal front of a stock culture was used to inoculate a fresh membrane-covered medium plate. Cultures were kept inverted in the dark and incubated at the standard growth temperature of 21°C.

Heat shock and radiolabeling. Label-containing plates were prepared by overlaying standard mimimal medium with 5 ml of medium containing ^{35}S-methionine (typically 6 μCi/ml; specific activity = 1100 Ci/mmole). The heat-shock response was induced by transferring plates supporting 4-day-old colonies to a 55°C incubator. Two methods of labeling heat-stress colonies were used: (1) pre-labeling, in which colonies and their subtending membranes were transferred to label-containing plates 15 min before being exposed to the high temperature stress, and (2) pre-shock, in which colonies were incubated at the heat stress temperature for 1 h, then transferred with their subtending membranes to prewarmed, label-containing plates and replaced in the high-temperature incubator. Control colonies, maintained at the standard incubation temperature for the duration of the experiment, were transferred to label-containing plates at the same time as test colonies. The duration of the exposure to high-temperature stress was typically 3 or 5 h.

Harvest and extraction. Colonies were harvested by scraping them from the surface of the membrane. Harvested colonies were weighed and quick-frozen with liquid nitrogen. The frozen mycelium was then transferred to a mortar and ground to a fine powder under liquid N_2. Cold extraction buffer (0.1 M K-phosphate, pH 7.0) was then added to the powdered mycelium, and the slurry was ground for 30 s [15]. The colony homogenate was then divided among 1.5-ml microfuge tubes and centrifuged for 10 min at 10,000 *g*. The supernatant was aspirated, pooled, and used as the crude extract. When the crude extracts were not used immediately, they were quickly frozen and stored at −80°C.

Analytical methods. The concentration of protein in the extracts was determined with Bradford reagent against a set of BSA standards [2]. The concentration of radiolabel in the colony extracts was determined by liquid scintillation counting. Two forms of polyacrylamide gel electrophoresis (PAGE) were utilized for this study: SDS–PAGE (12% T/3.44% C; [9]) and native gelatin-PAGE [10]. Native gelatin-containing PAGE separating gels (10% T/2.76% C) contained 0.3% (w/v) gelatin. Following SDS–PAGE, the gels were stained for total protein by the standard Coomassie blue method. Proteins containing radiolabel were visualized by fluorography using En^{3}Hance (E.I. Du Pont) and intensifying screens.

Native gelatin gels, containing separated proteases, were incubated in cold water for 15 min, cold citrate buffer (0.05 M citrate, pH 6.0) for 15 min, warm citrate buffer for 15 min, and warm citrate buffer for 2.5 h. The gels were then stained with Coomassie blue. Clear areas, where the gelatin had been degraded, indicated the presence of proteases.

Ubiquitin and ubiquitin-conjugated proteins were visualized by Western blot analysis [24]. Proteins were separated by SDS–PAGE and electrophoretically transferred onto a nitrocellulose filter. The filters to which the separated proteins were covalently bound were blocked with 1% (w/v) gelatin in Tris-buffered-saline + Tween 20 (TBST), incubated for 30 min in a solution containing a 1 : 100 dilution of an IgG fraction of rabbit anti-yeast-ubiquitin antibody (Sigma Chemical Co., St. Louis, Missouri), washed three times for 10 min each in TBST, incubated for 30 min in a solution containing a 1 : 2000 dilution of a goat anti-rabbit-IgG antibody that was conjugated to alkaline-phosphatase (AP), and washed three times in TBST for 10 min each. Filters were then incubated in AP-developing solution that contained 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in 100 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5.

Results and Discussion

The temperatures required to halt cell proliferation in the ascomycetes *Saccharomyces cerevisiae* and *Neurospora crassa* are 39°C and 45°C respectively [12, 18]. At 37°C, *Schizophyllum commune*'s growth rate was markedly increased, indicating that higher temperatures are required to induce the heat-shock response. Similarly, at 45°C radial growth continued, and there was a substantial increase in the production of aerial hyphae by the colony after 24 h at this temperature. Interestingly, consensus heat-shock elements (HSEs) have been found in the upstream regions of the *S. commune Sc3* and *Sc4* genes (F. Schuren and J. Wessels, personal communication). These genes code for hydrophobin proteins, which are deposited in hyphal walls and cause the production of aerial hyphae [27]. At 55°C there was cessation of growth of the *S. commune* colonies; however, transfer of the colonies back to lower temperatures, following a short exposure (ca 3 h) to 55°C, allowed resumption of growth after a short lag period. While a 3-h heat shock is long compared with the time necessary to induce the response in prokaryotes and yeast species, it represents only 10% of the normal doubling time of *S. commune* (dry wt. basis) at 21°C. As such, the exposure is comparable to that given to these organisms.

Transfer of 4-day-old *S. commune* colonies from 21°C to 55°C resulted in changes in the pattern of protein synthesis although the total amount of protein extracted from controls and heat-shocked colonies did not significantly differ (Fig. 1). In gels normalized for total radiolabel, it is apparent that there was a considerable increase in the synthesis of several new proteins. We have identified nine of these (those with the highest signal strength) as *S. commune* heat shock proteins. They are identified by their relative molecular weights as hsp114, hsp89, hsp75, hsp43.6, hsp38.5, hsp31.5, hsp21.5, hsp20.8, and hsp19.4. Including the nine major hsps, there are no fewer than 20 newly synthesized protein bands identifiable in pre-shocked samples. With minor differences in intensity, these proteins appeared in both pre-labeled and pre-shocked colonies.

Based on the high degree of conservation of hsps across taxa, two of the major hsps of *S. commune* can be assigned to hsp families with a fair amount of confidence: hsp75 to the hsp70 family and hsp89 to the hsp90 family. *Schizophyllum commune* hsp114 is approximately the same size as mammalian hsp110, which associates with nucleoli [26]; however, it is also possible that it is an analog to *Saccharomyces* hsp104, which may be involved in proteolysis [17].

Exposure of animal cell cultures to extreme heat shock (44°C) results in the synthesis of a 48-kDa peptide that does not appear when the cultures are shocked at 42°C [14]. Given the high temperatures used to induce heat shock in this study, hsp43.6 could be related to the animal cell hsp48. The 38.5-kDa and 31.5-kDa hsps of *S. commune* are similar in size to *N. crassa* hsp38 and hsp30. However, *Neurospora* hsp38 was reported to be induced only after conidiospores had begun to germinate [18]. *Neurospora* hsp30 has been found to be associated with mitochondria [19]. The remaining three, hsp21.5, hsp20.8 and hsp19.4, can be classified as low-molecular-weight hsps. Many low-molecular-weight hsps have been identified, but their specific functions remain a mystery [13, 25]. All of these identifications are based simply on similarities in size; more rigorous tests, however, such as antibody cross-reactivity, will be required to substantiate them.

A comparison of hsp114, hsp89, and hsp75 in the pre-labeled, heat-shock colonies and the pre-shocked, heat-shock colonies revealed a difference in the intensity of the bands (Fig. 1). The hsp114 and hsp75 bands were much more intense than the hsp89 band in colonies labeled prior to heat shock. When the colonies were incubated at 55°C for 1 h, prior to addition of the radiolabel, the three hsps had similar intensities. This suggests differential regulation of these genes. We hypothesize that the initial response to heat shock in *S. commune* results in synthesis of hsp114 and hsp75, followed by the synthesis of hsp89.

One of the best characterized heat-shock responses involves ubiquitin-mediated proteolytic pathways [6, 20]. Ubiquitin is a 76-amino-acid polypeptide with a primary structure that is highly conserved. Evidence suggests that proteins marked by poly-ubiquitination enter an ATP-dependent proteolytic pathway mediated by the multicatalytic protease or proteosome [7]. We exploited the highly conserved nature of ubiquitin by using an anti-yeast ubiquitin antibody to assess changes in the patterns of protein ubiquitination as a result of the transfer

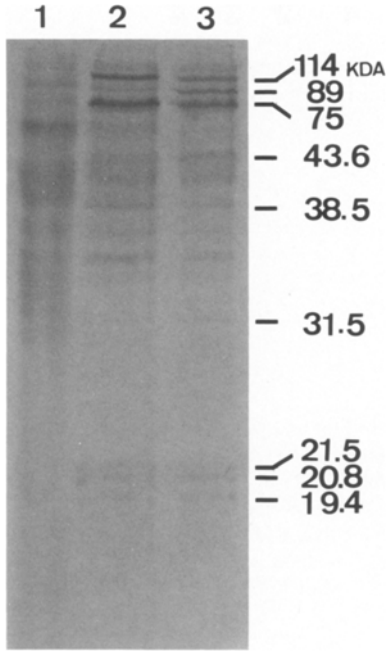

Fig. 1. Synthesis of heat shock proteins by *Schizophyllum commune*. Proteins were labeled in vivo with ^{35}S-methionine for 15 min prior to transfer to 55°C for 3 h (pre-labeled; lane 2) or were incubated 1 h at 55°C prior to transfer to ^{35}S-methionine medium for an additional 2 h at 55°C (pre-shocked; lane 3). Lane 1 = control transferred to label-containing plates and maintained at 21°C. Loads were normalized for total radioactivity.

of *S. commune* colonies to high temperature (Fig. 2). Although high-molecular-weight proteins were not very well resolved with this particular combination of gel size and acrylamide concentration, high-molecular-weight conjugates (HMWC) appeared to be less abundant in the heat-shock colonies than in the control colonies. Changes in lower-molecular-weight ubiquitin–protein conjugates owing to heat shock, were more easily discerned. Most apparent was the presence of a ladder of lower-molecular-weight ubiquitinated conjugates, the largest of which (M_r = 27.5 kDa) was present in highest concentration. The rungs of the ladder decreased in apparent intensity and molecular weight by increments of approximately 3 kDa.

At present, the best explanation for the ladder is that the bands are unrelated ubiquitinated conjugates. Ubiquitin plays a role in targeting proteins for degradation via the multicatalytic protease. Thus, it is possible that the bands are the result of proteolysis of the ubiquitin–protein conjugates and could account for the apparent decrease in the quantity of high-molecular-weight conjugates seen in the heat-shock extracts. This would mean that the protease

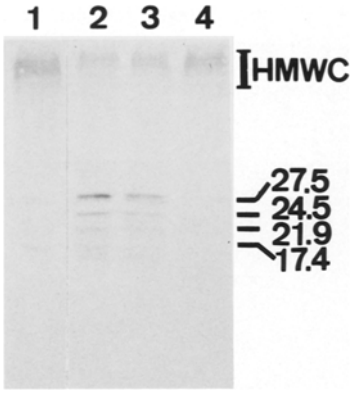

Fig. 2. Western blot analysis of extracts from control and heat-shocked colonies probed with rabbit anti-ubiquitin and anti-rabbit IgG-alkaline phosphatase conjugates. Lane 1 = 21°C control colony; lane 2 = heat-shocked colony; lane 3 = heat-shock colony extract incubated for 1.5 h at 25°C prior to electrophoresis; mixture of heat-shocked and control colony extracts incubated for 1.5 h at 25°C prior to electrophoresis.

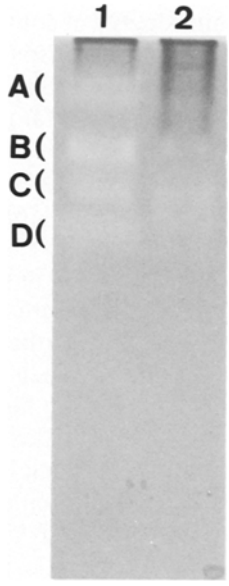

Fig. 3. Native gelatin-containing polyacrylamide gel of extracts from control and heat-shocked colonies showing activity of separated proteases against gelatin. Lane 1 = control; lane 2 = heat shock.

degrading these conjugates (perhaps the multicatalytic protease) was proceeding by cleaving 3-kDa fragments of the target protein. The accumulation of the fragments suggests that a portion of the subsequent proteolytic pathway was inactivated during heat shock. In a mixing experiment, in which extracts of heat-shock colonies were incubated 1.5 h at 25°C with extracts from control colonies prior to Western analysis, the ladder banding pattern disappeared. The ladder remained visible, but somewhat decreased in intensity, when the heat-shock extract was incubated alone (Fig. 2).

Schizophyllum commune produces numerous proteolytic enzymes that can be detected in gelatin-containing native polyacrylamide gels [10]. Heat shock decreased total proteolysis in gelatin gels (Fig. 3). While four banding areas, labeled A, B, C, and D, were visible with this particular gel size and acrylamide concentration, they represented the activity of many more than four proteases (Lilly, unpublished data). Proteases responsible for the appearance of banding area A were active in the control colonies but not in the heat-shock colonies. These proteases are regulated by nitrogen availability and nitrogen source; both nitrogen starvation and protein nitrogen sources lead to their increased activity [10, 11; Tilley and Lilly, unpublished data]. In an experiment in which colonies were transferred to low-nitrogen plates 3 h before being exposed to high-temperature stress conditions, there was an intermediate effect of the two stress responses (Fig. 4).

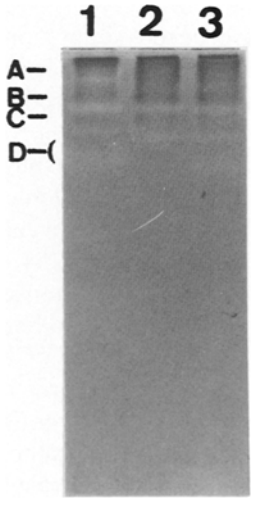

Fig. 4. Native gelatin-containing polyacrylamide gel of extracts of colonies exposed to the combined stresses of nitrogen deprivation and heat shock. Colony transferred to nitrogen-deficient medium for 6 h at 21°C; lane 2 = colony maintained on minimal medium heat shocked for 3 h; lane 3 = colony transferred to nitrogen-deficient medium for 3 h at 21°C and then heat shocked for 3 h.

The activity of proteases responsible for banding areas B, C, and D was also decreased because of heat shock. Whether this overall reduction in activity occurs with all proteases or is the result of the loss of a particular few proteases in each banding area is unknown. The application of both nitrogen

and heat stress also resulted in an intermediate response for those proteases corresponding to banding area D.

The response of *S. commune* colonies to heat shock includes changes in colony morphology, production of putative heat-shock proteins, changes in protein ubiquitination, and alteration of protease activity. Further analysis of the relationship between heat shock and proteolysis will lead to a greater understanding of the regulation of proteolytic enzymes in filamentous fungi.

ACKNOWLEDGMENTS

This work was supported by grants from the Grants and Research Funding Committee of Southeast Missouri State University and from the National Science Foundation (DCB-9004085) to WWL.

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