Gene expression of 70 kDa heat shock protein of *Candida albicans*: transcriptional activation and response to heat shock

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CaHSP70 (70 kDa heat shock protein) is a highly immunogenic protein of *Candida albicans*. We have studied heat shock-induced expression of the CaHSP70 gene under germ tube-inductive and non-inductive conditions. The CaHSP70 upstream regulatory region was cloned and sequenced. It contains at least three heat shock elements (HSEs), specific DNA sequences that are bound by the heat shock transcription factor (HSF), and one stress response element (STRE), which is an upstream activator sequence (UAS) that causes transcription activation under stress. The binding of HSF to HSE in the CaHSP70 promoter region is constitutive, although the mobility of protein/DNA complexes is altered after heat shock. The CaHSP70 promoter was cloned into a lacZ reporter plasmid, and was able to respond to heat shock in *C. albicans* as well as in *Saccharomyces cerevisiae*.

**Keywords** *Candida albicans*, heat shock, promoter

Introduction

*Candida albicans* is a dimorphic pathogenic fungus that has emerged as a leading cause of opportunistic infections in immunocompromised patients [1–4]. Although the importance of natural and adaptive immunity in the anti-*C. albicans* protection [5] is demonstrated, very few *C. albicans* immunogens have been discovered [6–9]. Identification of new immunogens as well as a better characterization of known antigens and how they are expressed would be of great value for understanding the mechanisms of the anti-*C. albicans* immune response. In previous studies, we have described the molecular cloning of the 70 kDa heat shock gene of *C. albicans* (CaHSP70, coding for a member of the 70 kDa heat shock protein family), the production and purification of a recombinant CaHSP70 protein and its immunogenic properties [9,10].

In this study, we focused our attention on CaHSP70 regulatory elements and heat shock-mediated expression of this gene in the different growth forms of *C. albicans*. This has been done to better understand the biological role of this heat shock protein and, in perspective, its potential for use as an immunomodulatory agent.

Materials and methods

**Microorganisms and growth conditions**

*C. albicans* ATCC 20955 (American Type Culture Collection, Manassas, VA, USA) was used throughout this study. Cells were pre-grown overnight at 28°C in YPD (1% yeast extract, 2% peptone, 2% dextrose), washed and inoculated at 22 and 37°C in basal saline medium [11] supplemented with 2.5 mM glucose. For germ-tube formation N-acetyl-d-glucosamine 4 mM was used as inducer instead of glucose. For autoradiography experiments, ³⁵S-labeled methionine was added to the medium. *C. albicans* CaI4 (Δura3::imm434/Δura3::imm434) (a gift from Dr W. A. Fonzi, Dept. Microbiology & Immunology, Georgetown University, Washington, DC, USA) was used as recipient strain for plasmid pRLV129 (this study) and *Saccharomyces cerevisiae* 15D (Mata, ade1, trp1, his2, leu2, ura3) (a gift from C. Wittenberg, Department of Molecular Biology, The Scripps Re-
search, La Jolla, CA, USA) was the recipient strain for plasmid pRLV121 (this study). They were grown and transformed as described previously [12,13]. *Escherichia coli* Y1090 (r{-}, m{-}, ΔlacU196, Δlon, supF, [pMC9]) (Stratagene, La Jolla, CA, USA) was the host strain for bacteriophage λgt11, whereas *E. coli* XL1 blue (endAI, hsdR17, supE44, thiI, recA1, gyrA96, relAI, Δlac, [F{', proAB, lacP2ZAM15, Tn10}] (Stratagene) was used as host strains for recombinant plasmids. *E. coli* cells were usually grown in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.00) or Luria–Bertani plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 7) supplemented, when necessary, with ampicillin (100 μg ml{−1}) or tetracycline (12.5 μg ml{−1}) (Roche Diagnostic SpA, Monza, Italy).

**Immunoblotting**

Whole cell extracts of *C. albicans* were obtained by cell breakage with 0.1 mm glass beads. The cell wall proteins were obtained by zymolyase treatment as previously described [11]. These extracts were resuspended in sample buffer, at ≈ 1 mg proteins per ml, boiled for 10 min and subjected to 5–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The electrophoresed materials (20 μg) were electroblotted onto nitrocellulose filters in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol. Filters were incubated with antibodies as detailed in single experiments. In all cases, nonspecific binding of antibodies to nitrocellulose was prevented by blocking the filters with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 h at room temperature. For extensive washing with PBS, bound antibodies were detected by suitable alkaline phosphatase-conjugates.

**Affinity purification of anti-CaHSP70/2 serum**

Hyperimmune serum against purified recombinant CaHSP70/2 was raised in CD2F1 mice (18–21 g) by four intraperitoneal injections, at weekly intervals, of 10 μg of the recombinant protein, in complete (the first two injections) and incomplete (the last two) Freund’s adjuvant. The titer of this serum was > 24,000, as determined by an immunoenzymatic assay with CaHsp70 used as coating antigen. Antiserum and CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech limited, Little Chalfont, UK) were mixed on rocker platform overnight at 4°C, centrifuged at 250 g for 10 min, incubated in 1 M ethanolamine (pH 8) for 5 h at 4°C, centrifuged, transferred to a column and equilibrated in PBS. The unbound proteins were washed with PBS and anti-CaHSP70/2 immunoglobulins were eluted with glycine–HCl buffer and stored at −20°C.

**Northern blot analysis**

Total RNA from *C. albicans* grown in germ tube inductive or non-inductive media at 22 or 37°C was isolated as described previously [9]. Approximately 5 μg of RNA per lane was run on denaturing 1.5% formaldehyde agarose gels, blotted onto nitrocellulose filters (Amersham Pharmacia Biotech) and hybridized with random-primed CaHSP70 [9] and rDNA gene probes. Hybridization and initial washing steps were done as described by Ausubel *et al.* [12], and the final stringent washing step was carried out in 0.1× sodium chloride/sodium citrate (SSC), 0.1% SDS at 70°C for 30 min. Filters were exposed overnight on XDA plus films (3M Italia SpA, Segré, Italy) with Trimax screens (3M) at −80°C.

**Primer extension**

Total RNA of *C. albicans* was extracted as described in northern blot analysis and quantified by spectrophotometric analysis. Approximately 50 μg were reverse-transcribed by the Moloney murine leukemia reverse transcriptase (Promega Corporation, Madison, WI, USA) with oligonucleotide Ca29 (5’-AATACCAA-CAGCTTTAGACATAATT-3’). The DNA was extracted using phenol–chloroform and after precipitation with potassium acetate 0.3 M and 2 vol. ethanol, the samples were centrifuged and rinsed in H2O. The fungal DNA was restricted with *EcoRI* enzyme, purified, ligated with dephosphorylated *EcoRI* λgt11 arms and incubated with *in vitro* packaging extracts.
(Boehringer), according to the manufacturer’s instructions. Recombinant phage particles were amplified by preparing plate lysates with *E. coli* strain Y1090. The amplified library (initial density: 40,000 plaques per 13 cm plate) was screened with the 700 bp *EcoRI* fragment of 5′ *CaHSP70* gene as probe.

**Cloning, sequencing and activity of pCaHSP70**

For molecular cloning of the λgt11 insert, *EcoRI* sites of the pbluescript (Stratagene), YEpl54 and YEpl56R (a gift from Dr C. Wittenberg) were used to give pRLV120, pRLV121 and pRLV124 (this study), respectively. Plasmid pRLV124 was digested with *ClaI* and *PstI*. The fragment containing the fusion pCaHSP70-lacZ was purified from agarose gel and ligated into *NarI* and *PstI* digested pKN4034 (a generous gift of Dr R. Cannon, unpublished results) vector to give pRLV129 plasmid. This recombinant plasmid contains the coding sequence for the fusion protein N-CaHSP70-beta galactosidase under the control of the CaHSP70 promoter. Double-strand dideoxy sequencing of recombinant plasmids was performed with the Sequenase Kit (USB, Cleveland, OH, USA) by using primers flanking the polylinker region of the vector and internal CaHSP70 sequences (EMBL database Accession no.: EMBL Z30210). The promoter sequence has been analyzed by using MACVECTOR 4.5.1 program (Oxford Molecular Group, Oxford, UK) and by visual inspection. It is homologous to the contig 6–2136 (bases 1314–1744 of the contig are homologous to bases –425 to 1 of CaHSP70 in Fig. 4) present in the *Candida* database (www.sequence.stanford.edu/group/candida). The Uracill strain of *S. cerevisiae* was transformed with pRLV121 by electroporation, as described previously [12]. The Uracill strain of *C. albicans* was transformed with pRLV129 as described previously [13]. The pCaHsp70 driven expression was monitored as β-galactosidase activity in *S. cerevisiae* cultures or as mRNA production in *C. albicans* cultures grown at different temperatures and in different cellular forms as specified in single experiments. Beta-galactosidase activity was measured as described elsewhere [12]. β-galactosidase cell wall linked activity was measured as the enzymatic activity of not-broken cells after extensive washing with reaction buffer.

**Band shift assay**

Ca24 (5′-TATTATTTCTTCCGGAATTTTGGTT- TAACAACT-3′) and Ca16 (5′-AGTGGTAAAA-C- GAATTTCGCCGAGAATAATA-3′) 32P-labeled oligonucleotides were denatured in 10 mM Tris–Cl pH 7.4, 0.25 mM EDTA, 100 mM NaCl for 5 min at 90°C and annealed at room temperature. Ca16/ Ca24 oligonucleotides were purchased from Amersham Pharmacia Biotech. OCT-1 (5′-AATTGCATGCTG-CAGGGTCACTCTAGGATCCATGCAATG-GATCCCGGGTACCGAGCTC-3′) double-strand oligonucleotide was a component of Band-shift kit (Amersham Pharmacia Biotech). Ca16/Ca24 and OCT-1 double-strand oligonucleotides were tested in gel mobility shift assay with *C. albicans* extracts by using Band-shift kit (Amersham Pharmacia Biotech) as described by the manufacturer.

**Results**

**CaHSP70 expression**

To assay for CaHSP70 protein production by *C. albicans* grown under germ-tube inductive and non-inductive conditions, an affinity-purified murine antiserum against the recombinant 21 kDa C-terminal fragment of the CaHSP70 protein was used in immunoblots with 35S-labeled whole cell extracts of the fungus. At least two reactive protein bands in the Mr region of 70 kDa were detected. Their reactivity was more intense when the fungal cell culture was shifted from 22 to 37°C, irrespective of the growth form (Fig. 1a). Autoradiography examination clearly demonstrated that proteins with molecular mass compatible with HSP70 were synthesized after heat shock (Fig. 1b).

The transcription of the fungal gene was studied by northern blot analysis of total RNA from cells undergoing temperature upshift (22–37°C) under germ-tube inductive and non-inductive growth conditions. The CaHSP70 hybridization signal was normalized against the ribosomal RNA. Figure 2 shows that CaHSP70 transcription was induced by heat shock and the induction was equal in both form of growth, demonstrating that protein production was due to CaHSP70 gene transcription rather than increased messenger translation.

**Mapping of transcription start point**

The specific oligonucleotide Ca29 was used for mapping the transcription startsite by primer extension (Fig. 3). Specific mRNA extension was observed in *C. albicans* cells after temperature upshift to 37°C for 30 min, irrespective of growth conditions. The length of the extended Ca29 primer corresponded to –120 ± 5 bp with respect to the start codon. We will hereafter refer to these sites as the CaHSP70 transcription startpoint. The same signal was detected in fungal culture without heat shock (22°C) although to a much lower extent. Other extensions, among which one with an intense signal...
corresponding to −60 bp, were observed at 22 and 37°C under non-inductive conditions.

Cloning of CaHSP70 promoter (pCaHSP70)

The cDNA probe used for screening the C. albicans genomic library was derived from EcoRI restriction of pRLV197, and represents the 5′ region of the CaHSP70 gene (27 kDa N-terminal coding sequence). Two independent 5 kb insert clones were isolated, subcloned into the EcoRI site of pbluescript, analyzed by restriction enzymes and partially sequenced. They shared the same sequence. The arbitrary 3′ insert sequence was identical to the probe sequence, and expanded into 5′ untranslated regulatory region upstream of CaHSP70 coding sequence (Fig. 4). We hereafter refer to this sequence as the CaHSP70 promoter (pCaHSP70). It shows the presence of one stress response element (STRE) and at least three heat shock elements (HSE). We named these sequences CaHSE1 (a 10 bp dyad symmetry element of two inverted 5 bp repeats), CaHSE2 (three 5 bp units arranged as direct repeats and spaced by 5 bp gaps) and CaHSE3 (five contiguous 5 bp modules). The locations of these HSEs are 175, 328 and 396 bp upstream of the CaHsp70 start codon.

pCaHSP70 driven-LacZ expression in S. cerevisiae

The 5 kb DNA fragment containing the pCaHSP70 was cloned into the EcoRI site of Yep354 to construct the recombinant plasmid pRLV121 and study LacZ expression driven by pCaHSP70. The plasmid was sequenced to confirm the in-frame construction, introduced into S. cerevisiae Ura− strain 15D, and was plated and selected on uracil dropout plates. The β-gal activity of transformant strain was analyzed after heat shock. As shown in Fig. 5, these cells constitutively expressed β-galactosidase but the enzymatic activity was almost doubled in response to a transient temperature upshift. Finally, the β-gal activity was much higher after longer exposure to 37°C (Fig. 5). Heat shock also induced enzyme expression by intact cells (data not shown).

pCaHSP70-driven-LacZ expression in C. albicans

The fragment containing the fusion pCaHSP70-lacZ was cloned into the NarI and PstI digested pKN4034 vector to give pRLV129. The plasmid was sequenced to confirm the construction and introduced into C. albicans CaI4 strain. As shown in Fig. 6 the CaHSP70-lacZ mRNA was induced after temperature upshift of the culture to 37°C.
Transcription factor binding activity

To search for heat shock transcription factor (HSF) binding to the CaHSP70 heat shock element 1 (CaHSE1), an assay was devised whereby crude fungal extracts were mixed with $^{32}$P labeled CaHSE1-containing double-strand oligonucleotide (Ca16/Ca24) and subjected to non-denaturing acrylamide gel electrophoresis. Ca16/Ca24 consensus sequence sited 180 bp upstream of CaHSP70 start codon. As shown in Fig. 7, HSF was able to bind CaHSE1 both in 22 and 37°C cell extracts, but the mobility of HSF/HSE1 complexes was reduced after heat shock suggesting a conformational change in the transcription factor/DNA complexes (Fig. 7, lanes 1, 2 and 3). The specificity of the reaction was demonstrated by using unlabeled target Ca16/Ca24 oligonucleotide (lanes 4 and 5) and unlabeled unrelated OCT-1 oligonucleotide (data not shown).

Discussion

Upon exposure to high temperature and certain other environmental stresses, all cells synthesize characteristic and highly conserved proteins collectively designated as heat shock proteins encoded by the so-called heat shock proteins.
genes [14]. The regulatory region of eukaryotic heat shock genes contains at least one copy of HSE, a cis-acting heat shock control element. This is a highly conserved DNA sequence that mediates rapid heat-dependent transcription activation [15,16]. It consists of a variable number of 5 bp building units (nGAAAn) arranged in alternating orientation every five nucleotides [17,18]. Heat shock promoters from different heat shock genes contain 2–8 repeats often separated by 5 bp of unrelated sequence [17]. The basic repeating units of heat shock promoters are binding sites for HSF, the heat shock transcription factor and as few as two inverted 5 bp units are able to bind homotrimeric HSF in vitro and in vivo [19].

Fig. 5 Activity of pCaHSP70 in Saccharomyces cerevisiae. β-Galactosidase activity of pCaHSP70-LacZ driven expression in S. cerevisiae cells grown at 22°C (■), heat shocked at 37°C (▲) or shocked at 37°C for 1 h and then shifted to 22°C (★). Time in hours and β-gal activity in arbitrary Miller Units.

Fig. 6 Activity of pCaHSP70 in Candida albicans. pCaHSP70-lacZ driven expression in C. albicans cells growing at 22°C (▲) or shifted up to 37°C (★) were analyzed by northern blot and normalized against rRNA.

Fig. 7 Heat shock transcription factor (HSF) binding to CaHSE1. Crude fungal extracts (5 μg) from cells grown for 30 min at 37°C (lanes 2 and 4) and at 22°C (lanes 3 and 5) were mixed with 32P-labeled CaHSE1-containing double-strand oligonucleotide (Ca16/Ca24) in presence (lanes 4 and 5) or absence (lanes 2 and 3) of 100-fold excess of cold Ca16/Ca24 oligonucleotide, and subjected to non-denaturing acrylamide gel electrophoresis as detailed in Materials and Methods. Unbound oligonucleotide was run in lane 1 as control.

Among eukaryotic cells, animal and fungal cells have a somewhat differing regulation of heat shock genes. In mammalian, Drosophila melanogaster and Schizosaccharomyces pombe cells, trimerization and change in the DNA binding properties of the HSF [19–23] mediate heat shock transcription activation. In Saccharomyces cerevisiae and Kluyveromyces lactis, HSF is constitutively trimeric and DNA-bound in an inactive form. Induction of transcription by heat shock is mediated by conversion of this HSF inactive form to a DNA-bound transcriptionally active form, probably by means of phosphorylation [24–28].

In a previous study, we described the isolation and sequencing of a C. albicans cDNA coding for a heat shock protein of 70 kDa [9]. The recombinant protein (CaHSP70) was produced in E. coli and purified by
metal chelate affinity chromatography. It was efficiently recognized in immunoblots by an anti-hsp70 monoclonal antibody and by sera of normal human subjects. Moreover, experiments with cultured peripheral blood mononuclear cells from the same subjects showed T-lymphocyte proliferation in response to the recombinant protein [9,10]. Finally, CaHSP70 was highly immunogenic in mice [10]. All this demonstrated the elevated immunogenicity of CaHSP70, as also expected of this protein family [29]. To clarify the biological role of this protein in the host/Candida relationship, we undertook a series of experiments primarily aimed at understanding regulation of CaHSP70 gene expression.

The expression of CaHSP70 was constitutive and a rapid transient induction was observed within 1 h after heat shock followed by a basal constitutive expression upon prolonged exposure to high temperature (1–3 h at 37°C). Under these experimental conditions, similar patterns of expression were observed in C. albicans under germ tube-inductive or non-inductive conditions, showing that CaHSP70 expression was unrelated to the morphology and was merely a response to the temperature stress. CaHsp70 protein was also induced by heat shock as demonstrated by Western blot analysis. Moreover, proteins with a molecular mass compatible with Hsp70 were newly synthesized after heat shock as shown by autoradiography of 35S-labeled cell extract.

The transcription start point was identified by primer extension. Specific mRNA has been detected in C. albicans cells after temperature upshift to 37°C, irrespective of growth form, and without heat shock (22°C), although at a much lower extent. In germ tube inductive conditions, the CaHSP70 mRNA was represented by a single transcript, whereas other extensions have been observed at 22 and 37°C (under non-germ tube-inductive conditions) including an intense 60 bp signal. The differences among the transcripts could originate from the different degrees of stability of CaHSP70 messenger under the various selected growth conditions. Alternatively, they could arise from different initiation of transcription or from changes in the specific secondary structure of mRNA. The different patterns of transcription could cause different patterns of CaHSP70 translation, but this was not analyzed here.

To identify the regulatory elements of CaHSP70 expression, we cloned and sequenced the CaHSP70 promoter. Three HSE and one STRE element were identified in pCaHSP70. Absence of a T residue at position 1 of all the CaHSP70 nGAAn sequences is of relevance. Indeed, the presence of this nucleotide in only one of the yeast HSE pentanucleotide repeats located upstream of the HSP70 gene has been found to cause a dramatic reduction of heat shock expression [26].

The binding of transcription factors to HSE sequences was demonstrated by a gel mobility shift assay. C. albicans crude cells extracts were incubated with double stranded oligonucleotides containing specific HSE binding sites. The binding of HSF to HSE is constitutive, but the mobility of the protein–DNA complex changes after heat shock. This finding is identical to the observation made with HSF–HSE binding in other normally non-pathogenic budding yeasts, in particular in S. cerevisiae and K. lactis [24,25,27,28], whereas the fission yeast S. pombe shows a pattern similar to that of higher eukaryotes [19,20,30]. This finding is not surprising, as C. albicans is evolutionarily closer to other budding yeasts than to fission yeasts. However, this is the first description of an HSP promoter in a human pathogenic Candida species.

CaHSP70 encodes a member of the Hsp70 protein family. The members of this family usually function as highly conserved immunodominant antigens of infectious agents. Preliminary data show that the CaHSP70 protein is ordinarily recognized as a B- and T-cell immunogen during experimental infection and also when C. albicans is growing commensally in humans. We have demonstrated, however, that CaHSP70, far from being a protective antigen, seems to facilitate infection by the fungus [10]. In this context, it is relevant to note that CaHSP70 is transcribed and expressed at a higher level at 37°C (body temperature) than at 22°C. Although the higher expression is transient, it occurs within the first hour after heat shock. This time correspond to an early, critical period in the host response during infection. The distinctive nature of host responses at this time is illustrated by our finding that IL-6 production in mice injected with CaHSP70 and its N-terminus/C-terminus fragments was highest in the first two hours post-infection [10]. We have also observed that pCaHSP70-driven β-gal expression in S. cerevisiae leads to the induction of CaHSP70 on the cell surface. This finding would indirectly confirm previous reports documenting the presence of HSP70 in the cell wall of S. cerevisiae and C. albicans [31,32]. Localization of CaHSP70 in the cell wall could be the factor explaining the high immunogenicity of this protein.

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