Molecular cloning, characterization and expression of the heat shock protein 60 gene from the human pathogenic fungus *Paracoccidioides brasiliensis*

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A gene encoding the heat shock protein (HSP) 60 from *Paracoccidioides brasiliensis* (*Pb*) was cloned and characterized. The *hsp60* gene is composed of three exons divided by two introns. Structural analysis of the promoter detected canonical sequences characteristic of regulatory regions from eukaryotic genes. The deduced amino acid sequence of the *Pb hsp60* gene and the respective cloned cDNA consists of 592 residues highly homologous to other fungal HSP60 proteins. The *hsp60* gene is present as a single copy in the genome, as shown by Southern blot analysis. The HSP60 protein was isolated from *Pb* yeast cellular extracts. N-terminal amino acid sequencing of HSP60 confirmed that the cloned *hsp60* gene correlated to the predicted protein in *Pb*. HSP60 expression appeared to be regulated during form transition in *Pb*, as different levels of expression were detected in *in vitro* labeling of cells and northern blot analysis. The complete coding region of *Pb hsp60* was fused with plasmid pGEX-4T-3 and expressed in *Escherichia coli* as a glutathione S-transferase-tagged recombinant protein. The protein reacted with a mouse monoclonal antibody raised to a human recombinant HSP60. Western immunoblot experiments demonstrated that the recombinant protein and the native HSP60 were recognized by sera from humans with paracoccidioidomycosis (PCM).

**Keywords**  HSP60 genomic and cDNA sequences, *Paracoccidioides brasiliensis*, protein expression

Introduction

*Paracoccidioides brasiliensis* (*Pb*), a thermally dimorphic fungus, is the etiological agent of paracoccidioidomycosis (PCM) [1,2]. The disease, a deep mycosis, affects many rural workers in Latin America. It is estimated to infect approximately 10 million people [3]. Although the natural habitat of *Pb* remains unclear, it is accepted that the conidia found in nature constitute the infective form. The conversion of the inhaled airborne propagules to the yeast form in human lungs establishes infection [4–6].

Heat shock proteins (HSP), also called stress proteins, are produced in large amounts by cells in response to rapid increases in temperature and other types of environmental stress. Stress-induced proteins protect cells and also participate in normal cellular functions [7]. They can be involved, for example, in protein translocation across cellular membranes, by acting as molecular chaperones [8]. It has been suggested that HSPs are also involved in the growth and differentiation of cells [9].

When entering the host from the environment, *Pb* is confronted by several stressful changes, including alterations in temperature. The *in vitro* fungal response to this environmental stress during form transition has been investigated [10]. The response includes the induction of
synthesis of HSP related proteins during conversion in isolates of Pb [10,11]. Despite the potential role of HSPs in Pb dimorphism, as described in other fungi [12,13], the only hsp gene from Pb that has been previously characterized is hsp70 [14]. The temperature-induced transition from mycelium to yeast is associated with an increase in the expression level of hsp70 mRNA and a transient accumulation of unspliced hsp70 mRNA transcripts.

HSPs are immunodominant antigens and major targets of host immune responses during infections [15]. Members of the HSP60 family, including those produced by pathogenic prokaryotes and lower eukaryotes, have been implicated as immunoprotective antigens, with exposure tending to immunize mammalian hosts against later infection [16–18]. For example, HIS-62 from Histoplasma capsulatum, a member of the HSP60 family, can immunize mice against development of histoplasmosis [18]. Peptides were generated to map specific regions of the protein, and a protective domain of the H. capsulatum HIS-62 was identified [19]. Hence, there is considerable interest in the potential role of members of the HSP60 family in modulating host immune responses.

In this study, we present the complete nucleotide sequence of the cloned hsp60 gene and the corresponding cDNA from Pb. The sequence is compared with others in related dimorphic fungal pathogens, and the antigenic properties of the protein are preliminary characterized after purification both from Pb itself and from a recombinant bacterial expression system.

Materials and methods

Study isolate and growth conditions

Pb isolate Pb01 (ATCC-MYA-826) has been investigated previously by our group and was used in this study [20–22]. The fungus was grown in Fava Neto’s medium [23]. The yeast and mould forms were cultured at 36 °C and 23 °C, respectively. The yeast cells were subcultured every 7 days and the mycelial form every 10 days.

Isolation of the hsp60 gene

A heterologous fragment, encompassing 600 bp, from nucleotides 856–1456 of the hsp60 gene from H. capsulatum [18] was used as a probe in the screening of a Pb genomic library [24]. Positive plaques were purified by three cycles of selection. Positive clones were characterized by restriction endonuclease mapping and Southern blot hybridization.

Pb hsp60 gene sequencing and sequence analysis

DNA purified from the putative genomic clone encoding hsp60 was digested with EcoRI and a 4.0-kb fragment that hybridized to the H. capsulatum probe was ligated in pBluescript II SK (Stratagene, La Jolla, CA, USA), according to the manufacturer’s protocols. Plasmid DNA was prepared using Qiagen kit (Qiagen, Hilden, Germany) and submitted to sequencing of both strands with a dye terminator and automated sequencer (Perkin–Elmer, Applied Biosystems, Norwalk, CT, USA). Nucleotide sequence analyses were performed with the Wisconsin Genetics Computer Group sequence analysis software package, version 7-0 [25]. The Blast program (National Center for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD, USA) was used to analyse nucleotide and deduced amino acid sequences and to compare sequences in the GenBank database [26].

Genomic DNA isolation

Pb yeast cells were harvested after seven days of culturing, washed and frozen in liquid nitrogen. The cells were broken by grinding with a mortar and pestle, and the genomic DNA was prepared by the cationic hexadecyl trimethyl ammonium bromide (CTAB) (Sigma, Aldrich, Inc., St. Louis, MS, USA) method [27], with minor modifications. To the cell powder was added 10 ml of extraction buffer (2% [w/v] polyvinylpolypyrrolidone (PVP) (Sigma), 1-4 M NaCl, 0-1 M Tris-HCl pH 8-0, 0-02 M ethylenediaminetetraacetic acid (EDTA) (Sigma), 2% [w/v] CTAB), and the mixture was incubated at 65 °C for 1 h. It was extracted with 50% chloroform/50% isooamyl alcohol [v/v] and precipitated with 100% ethanol. Subsequently, the DNA was subjected to RNAse I treatment, ethanol precipitation and resuspension in water.

Southern blot hybridization of genomic DNA

Southern blot analysis was performed according to standard procedures [28]. The Pb DNA was cut with selected restriction endonucleases. Digestion products were fractionated on a 1.0% [w/v] agarose gel and transferred to a Hybond-N-membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) after denaturation for 15 min in 0.5 M NaOH. An hsp60 probe, encompassing the entire sequence of the Pb gene, was labeled with a phosphatase labeling system (Amersham). Pre-hybridization and hybridization reactions were performed at 65 °C in a blocking reagent containing 50% [v/v] formamide. The blots were washed at 65 °C with 1×sodium saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate), 0.1% [w/v] sodium dodecyl sulfate (SDS).
cDNA synthesis and reverse transcription polymerase chain reaction (PCR)

Reverse transcriptase (RT)-PCR was performed using the RT-access–PCR kit (Promega, Madison, WI, USA). Total RNA was isolated with trizol according to the manufacturer’s instructions (Gibco BRL, Carlsbad, CA, USA) from yeast cells frozen with liquid nitrogen and disrupted by maceration. The RNA samples were treated with RNase free DNase I at 37 °C followed by phenol–chloroform extraction and ethanol precipitation [28]. Complementary DNA was synthesized from 1 μg of total RNA in the presence of synthetic oligonucleotide primers. The sense PCR primer was derived from the 5’ nucleotide sequence: [5’-GAATTC-GATGCAGCGAGCTTTTACT-3’], and the antisense primer was based on the 3’ end of the hsp60 gene: [5’-GCGGCCGCCTCTAGAACATACCCCCG-3’]. An EcoRI site and a NotI site (both underlined) were added to the sense and antisense primers, respectively, to facilitate cloning into plasmid pGEX-4T-3 (Amersham). The cDNA synthesis reaction was performed at 48 °C for 45 min. The PCR was subjected to an initial denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C (30 s), 47 °C (1 min), 68 °C (2 min) and a final extension at 68 °C (7 min). The RT-PCR product was gel purified using a Sephaglas Band Prep kit (Amer- sham), subcloned into plasmid pGEX-4T-3 and se- quenced as described above.

Expression of HSP60 in mycelium and yeast cells and during the dimorphic transition

Mycelium and yeast cells were labeled with 7.2×10^2 Bq of [35S]-l-methionine (Amersham) for 24 h in liquid S medium, as described [10]. The cells were also labeled during the form transition event. Yeast cells, grown at 36 °C, were transferred to liquid S medium and incubated at 23 °C with the radioactive precursor for 24 h. Mycelium, grown at 23 °C, was also transferred to the liquid medium and labeled at 36 °C for 24 h with [35S]-l-methionine. After incubation, the cells were collected by centrifugation at 10 000 g and processed as described above. The proteins were fractionated by two- dimensional gel electrophoresis.

Northern hybridization

Total RNA from Pb was isolated as described above. Northern hybridization was performed with 20 μg of total RNA from mycelium and yeast cells fractionated on a 1-2% [w/v] agarose–formaldehyde gel. A probe encompassing the entire hsp60 gene was labeled with [α-32P]-dATP using a random primers DNA labeling Kit RPN 1604 (Amersham).

Expression of hsp60 gene in Escherichia coli

The hsp60 cDNA was sub-cloned into the pGEX-4T-3 expression vector that had been restricted with EcoRI and NotI. This construct was transformed into E. coli XL1-Blue (Stratagene). Transformants were grown in 2×YT medium (1-6% [w/v] tryptone, 1% [w/v] yeast extract, 0.5% [w/v] NaCl) containing ampicillin (100 μg ml⁻¹), at 37 °C, in a shaking incubator. Expression of the fusion protein was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma).
Immunoblot analysis of the native HSP60 and of the recombinant fusion proteins

The yeast cellular extracts and the two-dimensional protein fractionation were performed as described above. After transfer to nylon membranes, the blots were reacted with a pool of 15 serum samples (1:500 diluted) obtained from individuals at the time of diagnosis of PCM, and representing several clinical forms of the disease. The transformed bacterial cells, expressing \( Pb \) HSP60, were lysed by adding buffer containing 0.2 M Tris-HCl pH 6.8, 4% [w/v] SDS, 0.2% [w/v] bromophenol blue and 2% [v/v] glycerol. The bacterial samples were fractionated on a 13% SDS-polyacrylamide gel according to Laemmli [30]. After transfer to membranes, the proteins were reacted with individual sera from patients with PCM, histoplasmosis, cryptococcosis and candidiasis. All sera were collected at the time of diagnosis from patients with confirmed active disease. The proteins were also reacted with a mouse monoclonal antibody raised to recombinant human HSP60 (Sigma). Human sera were pre-adsorbed with

Fig. 1  Nucleotide sequence of the \( hsp60 \) gene and the cDNA from \( Pb \). The introns are represented in lowercase. The putative TATA, CAAT, CAAG and HSE boxes are in bold. The solid double lines indicate conserved 5' and 3' consensus of the introns. The forward and reverse primers are underlined. The deduced amino acid sequence is shown below. The partial amino acid sequence from the native HSP60 is boxed with a dashed line.

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Results

Isolation and sequence analysis of the Pb hsp60 gene

A 600-bp genomic fragment from the *H. capsulatum* hsp60 gene was radiolabeled and used as a probe in the screening of a *Pb* genomic library. Six positive plaques were obtained after three cycles of selection and were then submitted to restriction mapping. The same restriction profile was obtained for all the clones. Accordingly, only one clone was chosen for further analysis. By mapping and restriction analysis, a 4.0-kb *Eco*RI fragment was subcloned and submitted to automated sequencing of both strands. The sequence analysis is shown in Figure 1. The DNA sequence of 2.42 kb included an open reading frame (ORF) that encoded a 592-amino acid polypeptide. Three exons separated by two introns were detected. The introns were flanked by 5′ GT and 3′ AG, which corresponded to published consensus sequences for splicing sites at intron–exon junctions in fungi [33]. Analysis of the 5′ noncoding region in the genomic clone revealed structural features typical of regulatory regions in eukaryotic genes. A sequence resembling a putative TATA box (AATAT) was identified 26 nucleotides upstream from the proposed translation start codon. Additional consensus sequences were present upstream from the TATA box. Two CCAAT boxes were identified at nucleotides 47 and 144 upstream from the start codon, as found in promoter regions of several eukaryotic genes [34]. CAAG motifs related to high expression levels of some fungal genes were seen at positions 290 and 406 [34,35].

A conserved sequence (NGAANNTTCNN) was found at nucleotide 270. This conserved sequence, generally found upstream from the TATA box [36], is homologous to the heat shock element (HSE) and may function in the regulation of heat shock genes [37]. A cDNA clone corresponding to the entire coding region of *hsp60* was obtained by RT–PCR. The cDNA sequencing confirmed the presence of two introns at positions 124 and 389 in the genomic DNA, and also confirmed the position of the initial methionine and the stop codon. The genomic and deduced amino-acid sequences of the *Pb hsp60* gene were deposited in GenBank (accession number AF059523).

Analysis of the deduced amino-acid sequence

The 1.77-kb cDNA contained a single ORF encoding a predicted protein of 62 kDa, with a pl of 5.44. The protein contains two putative glycosylation sites at positions 334 and 439 in the amino acid sequence. A chaperonin *cpn60* signature (AAVEEGILPGGG) is present at amino acids 443–454. The carboxy terminus of *Pb HSP60* is rich in methionine and glycine residues, a characteristic of HSP60 proteins [38]. Alignment of the deduced *Pb HSP60* with other sequences (Fig. 2) shows a high number of identical and conserved amino acids among the sequences. *Pb HSP60* is highly homologous to HSP60 from other fungus species, manifesting a 91% identity and 96% similarity with HSP60 from *Coccidioides immitis*, and 89% identity and 92% similarity with *H. capsulatum* HSP60 [18,39] (Table 1). Identity with HSP60 from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* was only 72% [40,41].

Genomic Southern blot analysis

A genomic Southern blot analysis was performed in order to estimate the number of *hsp60* gene copies. A fragment encompassing the whole cloned *Pb hsp60* gene was used as a probe. Genomic DNA from isolate *Pb01* was digested with six restriction enzymes. Each enzyme produced a restriction pattern that was consistent with a single copy gene (Fig. 3). The 4.0-kb *Eco*RI band (Fig. 3, lane b) corresponded to the fragment of the genomic clone from which the *Pb hsp60* gene had been isolated. The presence of more than one fragment in assays with *Hae*III and *Sac*I enzymes indicated the presence of internal restriction sites in the cloned fragment. For *Sac*I, one internal site was detected in position 1224 in the genomic sequence. For *Hae*III, nine internal sites were detected, most of them giving DNA fragments smaller than 400 bp (data not shown).

Table 1 Comparisons of *Pb* HSP60 amino acids and related sequences

<table>
<thead>
<tr>
<th>Sequence compared to <em>Pb</em> HSP60</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
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<tbody>
<tr>
<td><em>Ci</em> hsp60</td>
<td>91</td>
<td>96</td>
</tr>
<tr>
<td><em>Hc</em> HIS-62</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td><em>Ca</em> hsp60</td>
<td>73</td>
<td>86</td>
</tr>
<tr>
<td><em>Sp</em> hsp60</td>
<td>72</td>
<td>87</td>
</tr>
<tr>
<td><em>Sc</em> hsp60</td>
<td>72</td>
<td>86</td>
</tr>
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Amino acid sequences were analysed using FASTA and PILEUP programs of the Wisconsin Package Genetics Computer Group Inc (GCG) [25].

Hsp60 sequences were obtained as follows: *Coccidioides immitis (Ci)* [38]; *Histoplasma capsulatum (Hc)* [18]; *Candida albicans (Ca)* [12]; *Schizosaccharomyces pombe (Sp)* [39]; *Saccharomyces cerevisiae (Sc)* [40].
Fig. 2 Alignment of the deduced HSP60 sequence from Pb and reported sequences from other fungi. Coccidioides immitis (Ci), Histoplasma capsulatum (Hc), Candida albicans (Ca), Schizosaccharomyces pombe (Sp), Saccharomyces cerevisiae (Sc). Database accession numbers are Pb (AF059523), Ci (U91786), Hc (L11390), Ca (AF085694), Sp (Z66568), Sc (M33301). Asterisks indicate amino acid identity and dots represent conserved substitutions.
Identification and isolation of the native Pb HSP60 and NH$_2$ terminus sequencing

After two-dimensional electrophoresis of the Pb yeast cell lysates, the proteins were stained with Coomassie brilliant blue (Fig. 4a) or transferred to membranes. The blots were reacted against the monoclonal antibody to HSP60 (Fig. 4b). Two protein isoforms with a molecular mass of 64 kDa and pIs of 5.6 and 5.8 were shown to be reactive to the monoclonal antibody (Fig. 4b). The most abundant species, pI 5.8, was transferred to a PVDF membrane and submitted to amino acid sequencing. The obtained sequence (AHKELKFGVEARASLLKGID-TLA) matched the deduced amino-acid sequence from the cloned gene (Fig. 1).

Analysis of the expression of the native HSP60 in Pb cells

Isolation of the native HSP60 from yeast cells and mycelium was performed by two-dimensional electrophoretic analysis of newly synthesized proteins in stage specific forms and during dimorphic conversion. The cells were labeled with $[^{35}S]$-l-methionine and the expression of the HSP60 was analysed (Fig. 5). The HSP60 isoform with pI 5.8 was synthesized in lower amounts in mycelium than in yeast cells (Fig. 5, panels a and c). During conversion from mycelium ($23^\circ$C) to yeast form ($36^\circ$C), the expression of HSP60 increased (Fig. 5a, b). HSP60 expression decreased during the reverse process (Fig. 5c, d).

Northern blot analysis

The total RNA from mycelium and yeast cells was electrophoresed in a formaldehyde agarose gel and probed with the labeled genomic hsp60 fragment. A transcript of 2.1kb was detected in both cell types, but was much more abundant in yeast cells (Fig. 6, lane b) than in mycelium (Fig. 6, lane a).

Expression of hsp60 in E.coli

In order to express HSP60 in a prokaryotic system, the cDNA obtained by RT–PCR was subcloned into plasmid...
pGEX-4T-3 and induced with IPTG. Coomassie blue staining of the SDS– polyacrylamide gel electrophoresis (PAGE) revealed the expression of a 91-kDa protein in *E. coli* cells (Fig. 7c, f). The predicted molecular size of *Pb* HSP60 was 62 kDa, and the higher molecular mass of the expressed protein was due to the fusion with a 29-kDa GST protein (Fig. 7b). The recombinant protein exhibited strong reactivity to anti-HSP60 monoclonal antibody (Fig. 7f).

**Fig. 5** Analysis of HSP60 synthesis in *Pb* by two-dimensional protein analysis. Cells were incubated for 24 h with [35S]-l-methionine at the following temperatures: (a) mycelium at 23 °C; (b) mycelium at 36 °C; (c) yeast cells at 36 °C; (d) yeast cells at 23 °C. The cells were processed and the samples were submitted to electrophoresis according to O’Farrell [29]. Localization of HSP60 is marked with arrows. M→Y: mycelium to yeast cell transition; Y→M: yeast cell to mycelium transition.

**Fig. 6** *Pb hsp60* northern blot analysis. Total RNA from mycelium (a) or yeast cells (b) was fractionated in a formaldehyde agarose gel (1.2%) and hybridized to an *hsp60* genomic fragment.

Western blot analysis of human serum antibodies to native and recombinant *Pb* HSP60

After two-dimensional electrophoresis of *Pb* yeast cell lysates, the blots were reacted with sera from patients with a history of PCM (Fig. 8a) or with sera from non-immune individuals (Fig. 8b). Native HSP60 was reactive with sera from PCM patients. Immunoblot analysis of the recombinant HSP60 showed specific reactivity against sera from PCM patients (Fig. 8c, lanes 1 to 5). Sera from patients with other mycoses did not cross-react significantly (Fig. 8c, lanes 6 to 11).

**Discussion**

The evidence detailed above supports the contention that the *Pb* gene cloned in this study belongs to the *hsp60* family. Apart from the confirmatory features already mentioned, our *Pb* gene had an adenine base at position 1, in the putative HSE box, as is the case in *S. cerevisiae* and in *Drosophila* [42].
The predicted cloned HSP60 protein had a calculated pI of 5.44, with two putative N-glycosylation sites. The finding of two HSP60 isoforms in immunoblot assays in Pb yeast extracts is consistent with differential N-glycosylation attributable to the presence of these two sites. The sequence of the pI 5.8 isoform agreed perfectly with the major proportion of the predicted amino-acid sequence from the \( \text{Pbhsp60} \) gene. The predicted sequence, however, also included an additional 38 amino-acid residues at the NH\(_2\) terminus, which were not found in the sequenced protein. This 38 amino-acid region is rich in hydrophilic residues, making it compatible with mitochondrial signal sequences, as have been associated with other HSP60s [43,44].

The greater expression of HSP60 in yeast cells than in mycelium suggests that this protein could be necessary for \( \text{Pb} \)'s survival in host thermal conditions and that it may have a role in morphogenesis.

Immunoblot analysis of human IgG with the recombinant HSP60 showed that the protein was immunogenic for the majority of PCM patients (8/10, data not shown). HSP60 from several pathogenic microbes is antigenic. Recombinant HSP60 from \( \text{H. capsulatum} \) protects mice against intranasal challenge [18,19]. Recombinant HSP60 from \( \text{C. immitis} \) induces specific proliferation of T cells isolated from immunized BALB/c mice [39]. Such results suggest that the possible role of HSP60 in vaccination against PCM is worthy of investigation.

The observation that the native and recombinant HSP60s are not recognized by sera from normal human subjects or from patients with other mycoses is interesting. HSP60 proteins exhibit a high degree of homology among species, it is quite likely that the immune response is directed to the nonhomologous epitopes of this protein. Additional data must be obtained before the role of HSP60 in the immune response to \( \text{Pb} \) can be clarified.

In conclusion, the present study opens perspectives for analysing the extent to which immune responses to HSP60 can contribute to the development of protective immunity in PCM. The availability of the recombinant molecule also facilitates the process of defining the functional role of HSP60 in \( \text{Pb} \).

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Nucleotide sequence accession number: the sequence of \( \text{Pbhsp60} \) has been deposited in the GenBank database under accession number AF059523.

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Fig. 8 Analysis of immunological reactivity from native and recombinant HSP60 from *Pb*. Yeast extracts were analysed by two-dimensional electrofocusing. After transfer to membranes, the proteins were: (a) probed with a pool of human PCM sera (1:500 diluted); (b) reacted with non-immune sera. Localization of HSP60 is marked with an arrow. Numbers at the top are related to pH ranges in the first dimension and those on the left refer to molecular mass markers from second dimension (SDS–PAGE). (c) Representative immunoblot showing immunoreactivity of recombinant HSP60. Bacterial cell lysates from cells transformed with pGEX-4T-3-hsp 60 were separated by SDS–PAGE (13% gels) and transferred to nylon membranes that were cut in strips for assay with human serum. Blots were developed with individual sera from patients with: paracoccidioidomycosis (lanes 1 to 5); histoplasmosis (lanes 6 and 7); cryptococcosis (lanes 8 and 9); and candidiasis (lanes 10 and 11). Lane 12 shows immunoreactivity with monoclonal antibody to HSP60 and lane 13 shows reactivity with non-immune serum. The immunoreactive fusion protein band is indicated by the arrow.
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