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Review

Molecular chaperones in the *Paracoccidioides brasiliensis* transcriptome

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ABSTRACT. *Paracoccidioides brasiliensis* is a thermally dimorphic and a human pathogenic fungus. Our group has partially sequenced its transcriptome and generated a database of mycelial and yeast PbAESTs (*P. brasiliensis* assembled expressed sequence tags). In the present review we describe the identification of PbAESTs encoding molecular chaperones. These proteins, involved in protein folding and renaturation, are also implicated in several other biological processes, where the dimorphic transition is of particular interest. Another important issue concerning these proteins refers to their participation in the immunopathogenicity of infectious diseases. We have found 438 ESTs (184 in mycelium and 253 in yeast) encoding *P. brasiliensis* molecular chaperones and their co-chaperones, which were clustered in 48 genes. These genes were classified in families, corresponding to three small chaperones, nine HSP40s, 10 HSP60s, seven HSP70s, five HSP90s, four HSP100s, and 10 other chaperones. These results greatly increase the knowledge on *P. brasiliensis* molecular chaperones, since only eight of such proteins had been previously characterized.

Key words: *Paracoccidioides brasiliensis*, Heat shock proteins, Transcriptome, Molecular chaperone

INTRODUCTION

Paracoccidioides brasiliensis, a dimorphic and human pathogenic fungus, is the etiologic agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America. *In vitro*, this fungus can be cultivated in one of two inter-convertible forms, depending on the incubation temperature: mycelium at room temperature (26°C) and yeast at around 36°C. Two groups have partially sequenced the transcriptome of two different *P. brasiliensis* isolates (Felipe et al., 2003; Goldman et al., 2003). These groups have also analyzed the differences in gene expression between the mycelial and yeast forms of this organism. Our group has assessed this issue by two different approaches, the statistical analysis of gene expression based on expressed sequence tag (EST) counts (electronic subtraction) and a nylon cDNA microarray (Felipe et al., 2005). The *P. brasiliensis* mycelium to yeast dimorphic transition has been shown to be essential for the successful establishment of PCM (Salazar et al., 1988; Rooney and Klein, 2002). Lambowitz and colleagues (1983) have proposed that in *Histoplasma capsulatum*, a closely related pathogen, the morphological transition may be viewed as a heat shock event, followed by cellular adaptation to higher temperatures. Taking into account the importance of the heat shock response, in this review we present the data obtained in the search of PbAESTs (*P. brasiliensis* assembled ESTs) encoding molecular chaperones in the *P. brasiliensis* transcriptome.

MOLECULAR CHAPERONES AND THE HEAT SHOCK RESPONSE

In 1972, Christian Anfinsen received a Nobel Prize for his studies on the folding of polypeptides (Anfinsen, 1973), demonstrating that protein tri-dimensional structure is determined by the amino acid sequence, being readily adopted by unfolded proteins. Although this phenomenon may be true for most of the known proteins, this is not always the case, especially in the dynamic and protein-crowded intracellular environment.

The folding of newly synthesized proteins *in vivo* requires the involvement of a family of proteins, called molecular chaperones (reviewed by Walter and Buchner, 2002), which have been found in all living organisms studied so far. Some of these proteins are essential to cell viability, as described by Feder and Hofmann (1999). In this context, Riezman (2004) proposed that, during heat stress conditions, the accumulation of denatured or aggregated proteins is responsible for the loss of cell viability, reinforcing the importance of molecular chaperones in these events.

In addition to their participation in protein folding, molecular chaperones play other important roles, such as: a) preventing denaturation of folded proteins; b) preventing the potentially lethal aggregation of unfolded proteins and solubilizing these aggregates; c) maintaining proteins in an active conformation, and d) transporting proteins across membranes and assisting protein degradation (Demand et al., 2001). Surprisingly, heat shock proteins (HSPs) have also been shown to elicit humoral and cellular immune responses against several pathogens, including fungi (Zügel and Kaufmann, 1999).

Unfolded or denatured polypeptides expose hydrophobic amino acid residues which were buried inside the core of the folded protein. This inactive form tends to interact with similar regions in other polypeptides, leading to the formation of aggregates. Molecular chaperones may act by two distinct mechanisms: i) binding to these exposed residues and thus preventing

aggregation or, ii) performing ATP-dependent cycles, which provide optimized protein folding conditions.

The molecular chaperones were first described as HSPs, because they are over-expressed in response to heat shock. Other environmental changes, such as oxidative (Moraitis and Curran, 2004), osmolar (Siderius et al., 1997) and cold (Kandror et al., 2004) stresses, also induce the expression of molecular chaperones. These observations lead to yet another name for the molecular chaperones: stress proteins.

The heat shock response has been extensively studied in *Saccharomyces cerevisiae* and other fungal models. The most comprehensive knowledge databases came from genome-wide gene expression studies using tools such as SAGE and microarrays (Causton et al., 2001; Steen et al., 2002; Enjalbert et al., 2003; Chen et al., 2003; Xue et al., 2004). Studies with *S. cerevisiae* and *Schizosaccharomyces pombe* revealed a general stress response, by which a set of genes is upregulated as a response to all sorts of environmental stresses. Causton and colleagues (2001) have proposed a model where the transcriptional activators Msn2/Msn4 induce the common response to environmental changes. In yeast, Msn2 and Msn4, along with the heat shock factor Hsf, are involved in the transcriptional control induced by heat shock (Estruch, 2000; Gacto et al., 2003). Msn2/Msn4 bind to promoter sequences called stress response elements, inducing the transcription of genes related to heat and other types of stress as well, while Hsf binds to a different promoter sequence, called heat shock element. The mechanism by which environmental changes activate these transcription factors is not fully understood. Msn2/Msn4 are controlled by multiple MAPK- and PKA-dependent signal transduction cascades (Winkler et al., 2002; Harrison et al., 2004) and Hsf responds to similar cascades, although an alternative hypothesis of negative feedback mediated by HSP70 has been also proposed (Harrison et al., 2004; Hahn and Thiele, 2004). Molecular chaperone-encoding genes are also regulated at post-transcriptional (Thomsen et al., 2003) and translational (Preiss et al., 2003) levels.

Another type of chaperone corresponds to the carbohydrate trehalose, which is produced at high levels in response to several stresses in *S. cerevisiae* and other organisms. This molecule stabilizes proteins and biological membranes, being thus named a chemical chaperone. Its synthesis and degradation is tightly controlled in response to heat stress (reviewed by Voit, 2003).

Molecular chaperones are classified, based on their molecular masses, in families ranging from close to 10 kDa to over 150 kDa. Various classification schemes and nomenclatures have been proposed (Sghaier et al., 2004). In this study we decided to adopt the classical molecular mass-based criterion. The molecular chaperones and co-chaperones found in the *P. brasiliensis* transcriptome are shown in Table 1.

SMALL HSPS

The small chaperones are a family of structurally unrelated molecular chaperones, sharing three common characteristics: i) a small monomeric molecular mass ranging from 12 to 43 kDa; ii) the formation of large oligomeric complexes and, iii) the presence of a moderately conserved central region, the so-called α-crystallin domain (Narberhaus, 2002). These oligomeric complexes bind to unfolded proteins, preventing their aggregation and insolubilization. For each substrate, morphologically distinct and defined complexes are formed (Stromer et al., 2003).

Table 1. *Paracoccidioides brasiliensis* assembled expressed sequence tags (PbAESTs) related to heat shock and their associated proteins**.**

PbAEST	Annotation (gene name)	Number of ESTs	
		M	Y
Other chaperones			
2074	14-3-3 protein (*bmh1*)	5	6
499, 5172	14-3-3-like protein (*bmh2*)	4	7
1150, 2503	Calnexin (*cne1*)	11	1
3328	Peptidyl-prolyl cis-trans isomerase (*cpr2*)	0	1
1492, 295	Peptidyl-prolyl cis-trans isomerase (*cpr3*)	22	20
702	Peptidyl-prolyl cis-trans isomerase (*cpr5*)	2	4
277, 742	Peptidyl-prolyl cis-trans isomerase (*cpr6*)	4	2
1309, 506, 3271	Peptidyl-prolyl cis-trans isomerase (*cpr1*)	8	13
2661	FK506-binding protein (*fpr1*)	0	1
1432	FK506-binding protein (*fpr2*)	2	1
Small HSPs			
4615	Heat shock protein (*hsp12*)	1	0
1028	Heat shock protein (*hsp26*)	3	2
375, 4994	Heat shock protein (*hsp30*)	6	46
HSP40			
2349, 836	DNA J (Hsp40) ortholog (*caj1*)	4	5
755	DNA J (Hsp40) ortholog (*djp1*)	5	2
1039	DNA J (Hsp40) ortholog (*hlj1*)	4	5
838	DNA J (Hsp40) ortholog (*mdj1*)	4	8
5342	DNA J (Hsp40) ortholog (*scj1*)	1	0
680	DNA J (Hsp40) ortholog (*tim44*)	1	2
471, 5694	DNA J (Hsp40) ortholog (*ydj1*)	1	2
1200	DNA J (Hsp40) ortholog (*zuo1*)	2	0
3359	Molecular chaperone (*jac1*)	0	1
HSP60			
269, 318, 415	60-kDa heat shock protein (*hsp60*)	1	11
23	T-complex subunit (*cct2*)	1	1
1079, 2214	T-complex subunit (*cct3*)	3	2
2121, 3099	T-complex subunit (*cct4*)	1	2
1906	T-complex subunit (*cct5*)	0	2
387	T-complex subunit (*cct7*)	11	1
3490	T-complex subunit (*cct8*)	0	1
663	T-complex subunit (*tcp1*)	5	5
2555	Prefoldin subunit, chaperonin co-factor (*gim3*)	0	2
1711	GroES chaperonin (*hsp10*)	1	2
HSP70			
99, 3148, 3534	Heat shock protein 70 ortholog (*kar2*)	0	4
3178, 4680, 5352	Heat shock protein 70 ortholog (*mdl2*)	1	2
2210	Heat shock protein 70 ortholog (*hsp70*)	17	13
783, 3098	Heat shock protein 70 ortholog (*ssb1*)	0	3

Continued on next page

Table 1. Continued.

PbAEST	Annotation (gene name)	Number of ESTs	
		M	Y
902, 3349	Heat shock protein 70 ortholog (*ssc1*)	0	5
849, 4647, 4884	Heat shock protein 70 ortholog (*sse2*)	13	14
3910	Heat shock protein 70 ortholog (*ssz1*)	1	0
HSP90			
1824	High copy HSP90 suppressor (*aha1*)	0	2
1656, 4381	Heat shock protein 90 (*hsp90*)	15	25
1789	Hsp90 associated co-chaperone (*sba1*)	19	10
3752, 4104, 5192	Activator of Hsp70 and Hsp90 chaperones (*sti1*)	1	2
4541	Hsp90p co-chaperone (*cdc37*)	0	1
HSP100			
166	ClpB protease (*clpb*)	0	2
207, 3317	LON protease (*lon*)	0	3
179, 586, 2927	Heat shock protein (*mcx1*)	3	4
1250, 521	ClpA protease (*clpa*)	1	5

The sequences from *S. cerevisiae* (www.mips.gsf.de) were aligned with the set of PbAESTs. The ortholog name was based on the nomenclature used for *Saccharomyces cerevisiae*, with exception of the genes previously described in *Paracoccidioides brasiliensis*.

Three small chaperones were detected in the *P. brasiliensis* transcriptome. HSP12 is a cell membrane chaperone involved in response to different stresses and in cell adhesion (Zara et al., 2002). The PbAESTs 375 and 4994 encoding the molecular chaperone HSP30, showed the highest number of ESTs among the chaperones, with 6 and 46 ESTs generated from mycelium and yeast cDNA libraries, respectively. In *S. cerevisiae*, this gene encodes an integral plasma membrane chaperone which downregulates the activity of a plasma membrane H(+)-ATPase under stressful conditions (Piper et al., 1997).

HSP40

The HSP40 or DnaJ family is defined by the presence of a highly conserved J domain of approximately 78 residues. These proteins have their main role as HSP70 co-chaperones, even though a few examples of direct chaperone activity have been reported. HSP40 is required for the efficient binding of the target protein to HSP70 through the stimulation of HSP70 ATPase activity (reviewed by Fink, 1999).

Barros and Puccia (2001) have reported the sequencing of a genomic region with a gene encoding the HSP100 PbLON, and an adjacent partial sequence named PbMDJ1, an HSP40 family member. These authors have also shown that the PbMDJ1 mRNA is upregulated in heat shock and during the first 24 h of the mycelium to yeast dimorphic transition, being downregulated in the transition from yeast to mycelium. The results of the expression analyses of PbMDJ1-encoding PbAEST, did not reveal a differential expression pattern, since the elec-

tronic subtraction assay with four mycelium ESTs and eight yeast ESTs, showed a statistically non-significant P value of 0.059. Moreover, the nylon cDNA microarray analysis also did not show a differential expression profile for this gene (Felipe et al., 2005).

We have found eight other HSP40-encoding PbAESTs, including Ydj1, the most studied of all yeast HSP40s. Ydj1 interacts with and modulates the activity of Ssa1 cytoplasmic product (an HSP70). Some PbAESTs corresponding to the specialized proteins of the HSP40 family such as zuotin, a DNA-binding protein, and tim44, which facilitates the import of proteins into the mitochondria, are among these PbAESTs.

HSP60

The HSP60 family members, also called chaperonins, form a complex in the cytosol of prokaryotes and eukaryotes, as well as in several eukaryotic organelles. In prokaryotes, 14 GroEL and seven GroES subunits form this complex. The GroEL subunits adopt a barrel-shaped structure with a hydrophobic interior surface, where unfolded polypeptides are bound. The GroES subunits form a lid in the barrel, which then acquires a hydrophilic surface and releases a partially folded polypeptide in an ATP-dependent manner. Several of these cycles are necessary to completely fold a protein. Two similar complexes are found in eukaryotic mitochondria and chloroplasts, consisting of HSP60 and HSP10, homologs of GroEL and GroES, while the cytoplasmic chaperonin (named TCP-1, TRiC or CCT), is composed of eight different HSP60 family proteins forming a heteroligomeric complex. Each subunit is encoded by an independent and highly diverged gene, a phenomenon proposed to have been selected in order to cope with the folding and assembly of highly evolved proteins in eukaryotic cells (Kubota et al., 1995). An HSP60-based DNA vaccine with prophylactic and therapeutic properties is now under study in animal models (Bonato et al., 2004). Several fungal HSP60 homologs have been characterized, some of them with remarkable properties. In *H. capsulatum*, a cell wall HSP60 has been shown to be the ligand for a human macrophage receptor (Long et al., 2003). Deepe Jr. and Gibbons (2002) have shown that vaccination with Hsp60 from *H. capsulatum* induces a protective immune response in mice. Moreover, immunization of mice with recombinant HSP60 from *Coccidioides immitis* induces proliferation of T cells (Thomas et al., 1997).

A gene encoding a mitochondrial chaperonin has been characterized in *P. brasiliensis* and named HSP60. The gene's expression appeared to be regulated during the mycelium to yeast dimorphic transition (Izacc et al., 2001). The authors have also shown that an antibody present in PCM patient's sera recognized this protein. This protein immunogenicity was further explored using sera from 75 PCM patients and 94 control subjects. The results revealed a high sensitivity and specificity (97.3 and 92.5%, respectively) of this test, which was later proposed as a PCM serological test (Cunha et al., 2002).

We have found in our database genes encoding both HSP60 and HSP10 mitochondrial chaperonins. HSP60 seems to be more expressed than HSP10 (12 and 3 ESTs, respectively). We could not explain this difference in the expression levels, since both proteins are essential for the assembly of the mitochondrial protein folding machinery.

Finally, one last gene was classified in this family: the GimC complex subunit Gim3. This complex acts as an HSP60 co-chaperone for certain specific target proteins, such as actins and tubulins (Siegers et al., 2003). We have found all but one of the cytosolic HSP60 orthologs.

HSP70

The HSP70 family comprises several molecular chaperones with two characteristic conserved regions, the N-terminal ATPase and the C-terminal protein binding domain. In several organisms, HSP70 constitutes a very large protein family, being composed of multiple members. These proteins are present in most of the compartments inside the eukaryotic cell. HSP70 binds to small hydrophobic regions in unfolded polypeptides, thus preventing their aggregation. It also assists the target proteins' folding in an ATP-dependent manner.

In performing its task as a molecular chaperone, the HSP70s participate in several roles, which are important for dimorphic and pathogenic fungi. In *S. cerevisiae*, cytosolic HSP70s are required for the function of a cell cycle checkpoint (Gilbert et al., 2003). Antibodies against the *C. albicans* molecular chaperone Ssa1 are part of the serologic response in patients with systemic candidiasis (Pitarch et al., 2001). The same protein has been recently detected in the *C. albicans* cell envelope and was found to bind the human salivary fungicidal peptide histatin 5 (Li et al., 2003). The HSP70 molecular chaperones are essential for the fungicidal effect of the peptide, suggesting the great importance of this protein family in fungi. HSP70s have also been shown to be a major allergen of the yeast *Malassezia sympodialis* (Andersson et al., 2004), and major antigens in several other pathogens (Maresca and Kobayashi, 1994).

In *P. brasiliensis*, the HSP70 family corresponds to the most extensively studied HSP, with four members already described. The HSP70 gene encodes a cytosolic HSP70 ortholog similar to budding yeast's Ssa proteins. The HSP70 mRNA was shown to be differentially expressed in the yeast form of *P. brasiliensis* (Silva et al., 1999), as later corroborated by Goldman et al. (2003). Two other HSP70 genes have been cloned, characterized and reported by Florez and colleagues (2003). These genes' expression level rises during the mycelium to yeast dimorphic transition.

Another HSP70 ortholog described in *P. brasiliensis* was the 87-kDa HSP, first found as the target for a monoclonal antibody, which in an inhibition-ELISA diagnostic test confirmed its specificity against the *P. brasiliensis* 87-kDa HSP (Gomez et al., 1997, 1998). The characterization of the corresponding HSP87 gene sequence showed high similarity to HSP70 sequences (Diez et al., 2002, 2003).

Seven HSP70 orthologs have been identified in our database. The PbAEST 2210, which was assembled from 30 ESTs, encodes the previously described HSP70 (Silva et al., 1999) and was highly expressed. The other ortholog-encoded proteins were associated with the ribosome and were located in the cytoplasm, endoplasmic reticulum and mitochondria.

HSP90

HSP90 is a specialized ATP-dependent molecular chaperone present both, in bacteria, with the name of HtpG, and in eukaryotes. Unlike HSP70 or chaperonins, HSP90 assists the folding of a select set of proteins, primarily related to signal transduction. The most classical target proteins are steroid hormone receptors and kinases. Although the bacterial HtpG is not an essential gene, all disruptions of eukaryotic HSP90s have been proved lethal, a striking evidence for the importance of this molecular chaperone function (reviewed by Young et al., 2001).

The HSP90 protein is dimeric and is highly dependent on several co-chaperones to perform its function. Known co-chaperones in yeast include Sti1, Sba1, Aha1, Cdc37, Sse1, Cns1, and the immunophilins.

Several interesting reports involving fungi HSP90 have appeared in the literature in the past years. In *Podospora anserina*, the MOD-E HSP90 ortholog is involved in cell cycle regulation and sexual development (Loubradou et al., 1997). In *S. cerevisiae*, the inhibition of HSP90 function by an anti-HSP90 ribozyme promotes cell lysis, providing this protein as a therapeutic target (Sreedhar et al., 2003). In *C. albicans*, HSP90 was shown to be an immunodominant antigen, and that recovery from systemic candidiasis is closely related to the level of anti-HSP90 antibodies. With this in mind, a novel therapeutic strategy based on a human recombinant antibody to HSP90, which shows intrinsic antifungal activity and synergy with amphotericin B both, *in vitro* and *in vivo*, has been devised and is now in phase two of clinical trials (reviewed by Matthews and Burnie, 2004).

Goldman et al. (2003) showed an increased expression level HSP90 gene during the mycelium to yeast dimorphic transition, what is in accordance to our recent data.

We have found in our databases, PbAESTs corresponding to HSP90 itself and four of its co-chaperones: Aha1, Sba1, Sti1, and Cdc37. The 40 HSP90 ESTs are the second most abundant among all chaperones, indicating its high expression level. The co-chaperone Sba1 was also highly expressed, with 29 ESTs.

HSP100

Genes encoding molecular chaperones of approximately 100 kDa have been isolated and sequenced from various species. The gene products are termed Clp proteins because of their sequence similarity to *Escherichia coli* ClpA, which is thought to be involved in proteolysis regulation (Gottesman et al., 1990). Clp proteins are found in cytosolic and nuclear compartments, as well as in eukaryotic organelles and prokaryotic cells (Schirmer et al., 1994; Schmitt et al., 1995). High-molecular weight molecular chaperones have several functions, exemplified by ClpA-induced thermotolerance (Sanchez and Lindquist, 1990) and an HSP101-specific translational regulatory function controlled by nutrient status (Wells et al., 1998; Keeler et al., 2000).

Jesuino et al. (2002) have described a *P. brasiliensis* ATPase called PbClpB and demonstrated that this gene is preferentially expressed in the yeast form of the fungus. The ClpA protease (also named HSP104), mediates ATP-dependent unfolding of substrate proteins and targeting them into ClpP protease for degradation. The ClpA protein has also been shown to be upregulated during the mycelium to yeast transition and downregulated in the reverse transition (Goldman et al., 2003). The ClpA/P complex is required for the processive degradation of larger polypeptides in other organisms, but its function in *P. brasiliensis* is not known yet.

The other HSP100 found in *P. brasiliensis* is the PbLON protease (Barros and Puccia, 2001). This ATP-dependent mitochondrial protease is involved in the degradation of abnormal and short-lived proteins in *S. cerevisiae* mitochondria. Experiments have shown that the PbLON mRNA levels are increased after heat shock in yeast cells of *P. brasiliensis*. A third Clp protein described in yeast, named Mcx1p, locates at the matrix space of mitochondria. The protein Mcx1 may represent a molecular chaperone with non-proteolytic function in mitochondria (van Dyck et al., 1998). Only Mcx1 had not been previously described in *P. brasiliensis*.

OTHER MOLECULAR CHAPERONES

We have found some molecular chaperones, which do not fit into the main classes discussed above.

The immunophilins are proteins with peptidylproline cis-trans-isomerase activity (reviewed by Galat, 1993). They may function directly as chaperones or as HSP90 co-chaperones, linking HSP90-client protein complexes to the cytoskeleton. These proteins can be divided into cyclophilins and FKBPs, based on affinity to the immunosuppressant drugs cyclosporin and FK506. We have found two FKBPs and five cyclophilins in our databases.

The 14-3-3 proteins are part of a large family of conserved proteins which bind to chaperone diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors (reviewed by Fu, 2000). They are involved in cell cycle regulation, signal transduction, cell growth, and morphogenesis in *S. cerevisiae*. We have found both 14-3-3 fungal orthologs, Bmh1 and Bmh2.

We have also found PbAESTs encoding calnexin, an endoplasmic reticulum carbohydrate-binding protein (lectin), which controls, together with calreticulin, an endoplasmic reticulum protein quality control system. Calnexin binds glucosylated sugars attached to the unfolded or incompletely folded glycoproteins, ensuring a proper folding before it is exported from the endoplasmic reticulum (reviewed by Schrag et al., 2003).

CONCLUDING REMARKS

Important insights about *P. brasiliensis* have emerged from the PbAEST database. The presence of several highly expressed molecular chaperones encoding cDNAs stresses the importance of these proteins in fungal homeostasis. Among 6022 genes so far identified in *P. brasiliensis* transcriptome, a few can be pinpointed and further studied, in order to provide a better understanding of *P. brasiliensis* adaptability to changes in the environmental conditions.

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