

# Fungal heat-shock proteins in human disease

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## Abstract

Heat-shock proteins (hsps) have been identified as molecular chaperones conserved between microbes and man and grouped by their molecular mass and high degree of amino acid homology. This article reviews the major hsps of *Saccharomyces cerevisiae*, their interactions with trehalose, the effect of fermentation and the role of the heat-shock factor. Information derived from this model, as well as from *Neurospora crassa* and *Achlya ambisexualis*, helps in understanding the importance of hsps in the pathogenic fungi, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus* spp., *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Trichophyton rubrum*, *Phycomyces blakesleeanus*, *Fusarium oxysporum*, *Coccidioides immitis* and *Pneumocystis jiroveci*. This has been matched with proteomic and genomic information examining hsp expression in response to noxious stimuli. Fungal hsp90 has been identified as a target for immunotherapy by a genetically recombinant antibody. The concept of combining this antibody fragment with an antifungal drug for treating life-threatening fungal infection and the potential interactions with human and microbial hsp90 and nitric oxide is discussed.

## Introduction

Recent years have seen the progressive implication of a group of proteins referred to as heat-shock proteins (hsps) in human disease (Zugel & Kaufmann, 1999). These proteins have been characterized by their ubiquitous nature, high degree of amino acid homology, dominance in protein profiling and role as molecular chaperones (Lindquist & Craig, 1988).

Their definition as inducible by heat shock relates to their expression as part of a fundamental change in protein expression when an organism shifted from what was perceived as its normal ambient temperature to a higher or lower temperature. This situation has become more complex in that some hsps were produced constitutively; their expression has been linked to a whole range of noxious stimuli and not restricted to temperature change (Kaufmann, 1990). Where the minimum amount of hsps was expressed the temperature varied from 0 °C in arctic fish, where they were induced at 5–10 °C, to 50 °C in thermophilic bacteria, where the temperature had to be raised to 60 °C for induction (Daniels *et al.*, 1984; Cohen, 1992).

A further tenet has been the belief that a function defined for one protein in the cluster will hold for the homologue when that protein was detected in another organism. The model organism for bacteria was *Escherichia coli*, for yeasts *Saccharomyces cerevisiae* and for moulds *Neurospora crassa* and *Achlya ambisexualis*. This review will thus concentrate initially on the insights which have been obtained by studying the hsps of these fungi. The limitations of this approach are that these fungi rarely cause human disease and are not dimorphic, unlike some pathogenic fungi such as *Histoplasma capsulatum*; therefore, they cannot be considered solely as a suitable model. In dimorphic pathogens, a change in temperature is associated with a different phase of the life cycle; therefore, it is difficult to determine whether the expression of an individual protein was phase- or heat-induced. It also means that the roles of individual hsps are not confined to a heat-shock response but part of adaptation for survival in a mammalian host.

Hsps have been shown repeatedly to be immunodominant in infection. This is surprising, and against the concept that the immune response is targeted primarily against microbial-specific antigens. Homologues of the major hsps

occur in man. The immune system is, in effect, reacting vigorously to self-antigens. This has led to the concept of the immunological homunculus (Cohen, 1992) where an internal image of self-antigens occurs both in the thymus and in the periphery (Schwartz & Cohen, 2000). This natural autoimmunity is restricted to a few antigens of which the hsp's dominate. This natural autoimmunity is benign; it was hypothesized to be tightly controlled by anti-idiotypic cells, and has been exploited recently in the treatment of type 1 diabetes, where vaccination with a peptide derived from hsp60 delayed the onset of disease (Cohen, 2002).

This review will discuss the hsp's of the nonpathogenic fungi and of pathogenic fungi, including their role when the fungus causes human disease. We will comment on the potential of hsp's as the basis of active or passive immunotherapy and on therapies involving synthetic compounds which target hsp's. We will conclude with the idea of treating a fungal infection with a conventional antibiotic in combination with an antibody fragment targeted to a specific fungal hsp.

## Hsps and nonpathogenic fungi

### *Saccharomyces cerevisiae*

#### Hsp12

Hsp12 has been shown to be produced under conditions of high osmolarity, heat shock, ethanol and oxidative stress (Mtwisha *et al.*, 1998). Its expression coincided with entry into the stationary phase of growth and in mutants harbouring a defective adenylate cyclase. A detailed analysis of the promoter region revealed five repeats of the stress-responsive CCCT motif which were essential to confer wild-type expression (Varela *et al.*, 1995). Subsequently, expression has been shown to be controlled not only by a stress response but also by a glucose-dependent mechanism (de Groot *et al.*, 2000). Very low concentrations of glucose-6-phosphate (0.02%) triggered hsp12 repression in non-fermenting yeasts, where it served as a signalling molecule. The major transcription factors involved in the stress response were Msn2p/4p and Hspfp (Ivorra *et al.*, 1999) binding to the promoter elements. Glucose repression was independent of this mechanism (de Groot *et al.*, 2000). Biofilm formation was inhibited in Sardinia sherry strains of *S. cerevisiae* at the end of ethanol fermentation, when glucose was depleted in a strain with a point mutation in HSP12 (Zara *et al.*, 2002). Hsp12 is located in both the cytoplasm and the cell wall. A role in the increased flexibility of the cell wall, coupled with a pressure-resistant phenotype (barotolerance), has been suggested (Motshwene *et al.*, 2004).

#### Hsp26

Hsp26 is a major hsp in eukaryotic cells. In *S. cerevisiae*, it was absent in unstressed vegetative cells and was induced strongly by heat shock, stationary-phase arrest, nitrogen starvation and salt shock (Carmelo & Sa-Correia, 1997). It was not required for growth at high temperatures, nor for thermotolerance, spore development or germination (Bossier *et al.*, 1989). Transcription was regulated by basal repression during growth at normal temperatures and depressed when heat-shocked or grown at low pH (Carmelo & Sa-Correia, 1997). Hsp26 is 213 amino acids long and contains the highly conserved tetrapeptide sequence Gly--Val--Leu--Thr (Bossier *et al.*, 1989). The promoter contains evolutionary conserved heat-shock elements (HSEs) characterized as multiple continuous and involved repeats of the penta nucleotide nGAAn. These are the binding sites for the regulatory heat-shock factor (HSF). Increasing the number of HSEs led to only a moderately increased rate of accumulation of the mRNA responsible for hsp26. As few as two HSE-HSF complexes were sufficient to saturate HSFs target in the basal transcription state (Chen & Pederson, 1993).

Overexpression of hsp26 slightly increased thermotolerance (Bentley *et al.*, 1992) and occurred in cells lacking constitutive hsp70 (Unno *et al.*, 1998). Expression was also mediated via the transcription factors Msn2p and Msn4p, which bind to the stress-response element (STRE). These are zinc finger proteins found in the cytoplasm of cells which accumulate in the nucleus under stress conditions.

The expression of hsp26 in response to heat has been linked to induction by both HSE and STRE with an additive effect. The induction of hsp26 by carbon growth starvation, oxidative and osmotic stress were reduced severely in an Msn2/Msn4 double mutant, suggesting a pivotal role for STRE (Amoros & Estruch, 2001).

Hsp26 is a cytosolic protein that exists as a hollow shell of 24 subunits under physiological conditions (Haslbeck *et al.*, 1999; Friedrich *et al.*, 2004). It has been shown to act as a chaperone and bind non-native proteins in a co-operative manner. At elevated temperatures, the 24-subunit complex disassociated into dimers. The dissociation seems to be required for the efficient interaction of hsp26 with unfolding proteins, resulting in the formation of large regular complexes comprising hsp26 and the non-native protein (Stromer *et al.*, 2004).

#### Hsp30

Hsp30 was initially identified by its induction when *S. cerevisiae* entered the stationary phase of growth (Regnacq & Boucherie, 1993). It had an apparent molecular weight of 30 kDa and was induced by heat shock, ethanol concentrations (4–10% v/v) that affect growth and

fermentation rates, severe osmotic stress, weak organic acid exposure and glucose limitation (Panaretou & Piper, 1992; Piper *et al.*, 1997). Expression of this hsp correlated with a reduction in ATPase activity at the plasma membrane (Panaretou & Piper, 1992); this process was not induced by activation of HSF or through STREs (Seymour & Piper, 1999). Hsp30 acted by reducing the  $V_{max}$  of ATPase in heat-shocked cells and this effect was abolished in mutants of the ATPase lacking the carboxy-terminal 11 amino acids. Mutation of the threonine at amino acid 912 (which was a potential site for phosphorylation by a calcium-calmodulin-dependent protein kinase) also reversed this effect (Bralely & Piper, 1997).

Two homologues Mrh1p (67% identity) and Yro2p (78% similarity) to *S. cerevisiae* hsp30 have been described (Wu Ke & Aris, 2000). The localization of Mrh1p to the plasma membrane suggested that it participated in similar cellular processes to hsp30. The function of Yro2p is currently unknown.

### Hsp40

Sixteen hsp40s have been identified in *S. cerevisiae* by their sequence similarities to *E. coli* DnaJ (Table 1) (Yan & Christians, 1999). They have been subdivided into two classes (Johnson & Craig, 2001). Both have a signature J domain of approximately 70 amino acids, with significant conservation of amino acid sequence among evolutionarily diverse organisms. The J domain was present in seemingly random locations within the hsp40. Considerable evidence suggests that the J domain directs and orchestrates interactions with and recruits a specific hsp70 family member. Hsp40 is thought to change the conformation of hsp70 to a form that displays a higher affinity for its various substrates following ATP hydrolysis. The J domain contains a conserved HPD tripeptide motif. This area regulated the ATP hydrolytic cycle of hsp70, where it was proposed that the HPD motif formed a cleft at the base of hsp70. This promoted a relatively stable complex between hsp70 and

unfolded polypeptides. Hsp40s can bind unfolded polypeptides and transfer them to hsp70. The ATP hydrolysis induced a conformational change in hsp70 involving the closure of a lid structure that stabilizes the hsp70 peptide complex (Lee *et al.*, 2002). After the J domain, both classes have a glycine- and often a phenylalanine-rich area. They differ in that Class I molecules have a zinc finger-like domain and a conserved carboxyl-terminal domain which binds non-native proteins. The classic *S. cerevisiae* Class I molecule was Ydj1 and the *E. coli* equivalent was DnaJ. In Class II molecules, such as Sis1 in yeast and human Hdj1, the conserved terminal domain was retained, but the zinc finger-like region was replaced by a segment rich in glycine and methionine.

SIS1 was found to be an essential gene while the deletion of YDJ1 resulted in cells which grew poorly. Overexpression of SIS1 compensated for the slow growth but the reverse did not apply, so that the expression of SIS1 was seen as core to the cell function (Caplan & Douglas, 1991; Luke *et al.*, 1991). Sis1 was necessary for propagation of the *S. cerevisiae* prion (RNQ<sup>+</sup>), whereas Ydj1 was not (Lopez *et al.*, 2003). The control of this function was delineated to a short stretch within the glycine-phenylalanine area of Sis1, as single amino acid alterations caused defects in prion maintenance (Lopez *et al.*, 2003). The interaction between Sis1 and the hsp70 homologue Ssa1 has been studied in depth (Qian *et al.*, 2002). A model has been proposed in which the terminal 15 amino acids of Ssa1, containing the motif EEVD, acted as an anchoring function for hsp40 to deliver non-native polypeptides to hsp70 for folding. A glycine-rich linker in front of the anchoring motif has been perceived as generating the flexibility needed for Ssa1 to accommodate the stretched-out non-native polypeptides into the Ssa1 peptide-binding groove. Sis1 has been implicated in translation initiation, as mutations in ribosomal protein L39 complement for the loss of function mutations in Sis1 (Horton *et al.*, 2001).

The relationship between Ssa1 and Ydj1 has also been studied (Meacham *et al.*, 1999; Johnson & Craig, 2000). Ydj1 and Ssa1 have been shown to function in the biogenesis of  $\alpha$ -factor, the pheromone secreted by MAT  $\alpha$  cells. In order for efficient mating MAT a and MAT  $\alpha$  yeasts must produce their respective mating pheromones. These were synthesized as pro-forms and converted by peptidases to mature forms. Ydj1 promoted the post-translocation of prepro- $\alpha$ -factor across the endoplasmic reticulum such that a strain with a mutant Ydj1 exhibited a severe mating defect and a 90% reduction in  $\alpha$ -factor secretion. Axl1 was the catalytic protein involved in the cleavage of the amino terminus 14 amino residues that produced the mature  $\alpha$ -factor. Ydj1 functioned by promoting AXL1 mRNA accumulation and by facilitating the proper folding of Axl1. Axl1 was an *in vivo* substrate of the hsp70/Ydj1 chaperone system (Meacham

**Table 1.** Location of hsp40s and hsp70s in *Saccharomyces cerevisiae*

Location within cell	Hsp40	Hsp70
Mitochondria	Mdj1, Mdj2 and Jac1*	Ssc1 <sup>†</sup>
Cytosol	Ydj1, Sis1, Zuo1 and Djp1*	Ssa1, Ssa 2, Ssa 3 and Ssa 4*
Ribosome	–	Ssb1, Ssb2 and Pdr13* (or Ssz1) <sup>‡</sup>
Endoplasmic reticulum	Sec63, Scj1 and Jem1*	–

\*Yan & Christians (1999).

<sup>†</sup>Voisine *et al.* (1999).

<sup>‡</sup>Frydman (2001).

*et al.*, 1999). Ydj1 has also been implicated in presenting steroid receptors to the hsp90 pathway for folding (Johnson & Craig, 2000).

### Hsp60 and Hsp10: the mitochondrial chaperones

Hsp60 has been shown to be essential for growth at all temperatures in *S. cerevisiae* and is expressed at an increased level of two- to threefold when yeasts were grown at 42 °C (Hallberg *et al.*, 1993). It is the yeast equivalent of *E. coli* GroEL and promotes folding of proteins in the mitochondrial matrix (Cheng *et al.*, 1989). GroEL is composed of 14 monomers with two 7-mers as rings, so that substrate proteins can be isolated in a central cavity where they mature (Cheng *et al.*, 1990). Release is catalysed by the co-chaperone hsp10 in an ATP-dependent reaction.

Hsp10, the fungal equivalent of GroES, exists as a heptamer and is part of the mitochondrial protein-folding apparatus. It is involved in the sorting of certain proteins, such as the Rieske Fe/S protein passing through the matrix en route to the intermembrane space. Mutations in the region equivalent to the loop region of bacterial GroES lead to temperature sensitivity and reduced chaperonin binding (Landry *et al.*, 1999). In the presence of ADP, one molecule of hsp10 binds to hsp60 with an apparent K<sub>d</sub> of 0.9 nM and a second molecule binds with an apparent K<sub>d</sub> of 24 nM. In the presence of ATP, purified hsp10 and hsp60 refolded mitochondrial malate dehydrogenase (Dubaquie *et al.*, 1998). A temperate-sensitive mutant of hsp10 (P36H) reduced this ability at 25 °C and abolished it at 30 °C.

Hsp60 bound to unfolded Firefly luciferase, but it required the presence of hsp70 to assist efficient folding (Heyrovska *et al.*, 1998). Hsp60 was required to prevent the thermal inactivation of native dihydrofolate reductase by mediating ATP-dependent refolding at increased temperatures (Martin *et al.*, 1992).

The *in vitro* substrates of hsp60 and hsp10 have been shown to vary, with some proteins folded in the absence of functional hsp10 (Dubaquie *et al.*, 1997). The substrates had molecular weights varying from 15 to 90 kDa and could be subdivided into proteins that required both molecules, proteins that failed to fold after hsp60 inactivation but were unaffected by hsp10 inactivation and those affected more severely by hsp10 inactivation than pre-existing hsp60 inactivation. This demonstrated that hsp60 and hsp10 did not always act as a single functional unit *in vivo*. In mitochondria, the peptidyl-prolyl-isomerase, cyclophilin encoded by CPR3, has been shown to be important for folding newly imported preproteins when acting in concert with hsp60 (Voos & Rottgers, 2002). The integration of immunodeficiency virus type 1 into the nuclear genome was catalysed by the retroviral enzyme integrase. In yeast,

integration of this enzyme induced a lethal phenotype when a mutated hsp60 was co-expressed (Parissi *et al.*, 2001).

Hsp60 functionality has been studied by producing a series of yeast strains expressing hsp60 with carboxy-terminal deletions (Fang & Cheng, 2002). All the mutants interacted with hsp10 but were defective in their ability to refold denatured rhodanese. Other studies of mutant yeast strains, in which the level of hsp60 production was four times greater than wild-type cells, demonstrated that the ability to resist oxidative stress correlated with the level of hsp60. This was linked to the protection of iron/sulphur-containing enzymes from oxidative inactivation (Cabiscol *et al.*, 2002).

### Hsp70

Hsp70s have been described as a highly conserved family of at least 10 proteins with a molecular mass of approximately 70 kDa. They are composed of two functionally separate domains, one of 44 kDa responsible for ATP binding and the other of 18 kDa, which binds to the substrate peptide. The *E. coli* homologue is DnaK and ATPase activity is stimulated by DnaJ (*S. cerevisiae* hsp40). These proteins produced ATP-dependent stabilization of the hydrophobic regions in extended polypeptide segments. The cellular locations of *S. cerevisiae* hsp70s are shown in Table 1. The Ssa hsp70 molecule was essential for viability and could not be compensated for by Ssb activity (Craig *et al.*, 1994). The Ssa proteins were involved in the translocation of proteins across membranes and in the regulation of the heat-shock response. Ssa1 and Ssa2 were constitutively expressed, while Ssa3 and Ssa4 were expressed under stress conditions. Ssb1 differed from Ssb2 by four amino acids (Satyanarayana *et al.*, 2000) and from the Ssa family by approximately 37% (Lopez-Buesa *et al.*, 1998).

Ssa1 is the most studied cytosolic hsp70 and its interaction with Sis1 is discussed in the hsp40 section. Conceptually, the hsp40 homologue delivered non-native polypeptide to the hsp70 homologue which then stimulated the ATPase activity of hsp70 to fold the polypeptide (Qian *et al.*, 2002). This underwent repeated cycles of binding/release, so that the conformational change was local rather than global (Bukau & Horwich, 1998). Fes1, an endoplasmic reticulum protein, binds preferentially to the ADP-bound form of Ssa1 and promotes nucleotide release. Paradoxically, Fes1 inhibited Ydj1-mediated activation of Ssc1 ATPase (Kabani *et al.*, 2002).

The effect of Ydj1 and Ssa1 on the translocation of histidine-tagged prepro- $\alpha$ -factor into microsomes has been measured. Both molecules stimulated post-translational translocation by preventing aggregation and this required ATP hydrolysis by Ssa1 (Ngosuwan *et al.*, 2003). Ssa1 and Ssa2 have been implicated in the folding of ornithine trans-

carbamoylase (Kim *et al.*, 1998) and the transport of vacuolar hydrolase aminopeptidase I from the cytoplasm into the vacuole (Satyanarayana *et al.*, 2000). Ssa1 has been shown to be involved in regulating the stability of yeast AU-rich elements identified in the 3' untranslated regions of several mRNAs such as MFA2 and TIF51A (Duttagupta *et al.*, 2003).

Ssa2 has been identified as one of the ATP-binding proteins involved in the transport of fructose-1, 6-bisphosphatase into the vacuole for degradation when *S. cerevisiae* was shifted from high to low glucose (Brown *et al.*, 2000). Ssa4 concentrates in nuclei during yeast starvation, which is reversed when stationary cells are transferred to fresh medium (Chughtai *et al.*, 2001).

The Ssb ribosomal location has been correlated with a conserved residue in a peptide-binding cleft (valine 442) which tolerated a number of alterations revealing a high degree of flexibility (Pfund *et al.*, 2001). The Ssb1 and Ssb2 cross-link product, together with Ssz1 and the hsp40 homologue zuotin, form a chaperone triad acting on nascent chains emerging from the ribosome (Gautschi *et al.*, 2002). Ssa and Ssb have diverged functionally with regard to their roles in prion formation and propagation within yeasts. Ssb has been described as part of a proof-reading system with the aim of preventing the formation of protein aggregates (Chernoff *et al.*, 1999).

In mitochondria, there are three species of hsp70 encoded by the genes SSC1, SSQ1 and ECM10. Deletion of SSC1 was lethal under all conditions. Functions have included pre-protein translocation, protein folding and stress protection (Krimmer *et al.*, 2000).

The ATPase activity of Ssc1 was enhanced by Mge1, a homologue of the bacterial GrpE protein. Null mutations of MGE1 were lethal, and it was important in influencing the affinity of hsp70 to substrate proteins and in orchestrating ATPase activity (Voos & Rottgers, 2002).

The import of preproteins into the mitochondrial matrix was mediated by mitochondrial hsp70 working with the translocase subunit Tim44 of the inner membrane. This functionality was linked to an 18-amino-acid stretch in Tim44 (Geissler *et al.*, 2000) and the ATP-binding domain of hsp70 (Krimmer *et al.*, 2000). This system actively unfolded preproteins and shortened the lag phase of translocation (Lim *et al.*, 2001). It has been proposed that it operates by both trapping the preproteins passively (Geissler *et al.*, 2001) and pulling them through actively (Chiang *et al.*, 2003). The hsp40 homologue Mdj1 has been implicated in preventing recently imported proteins misfolding (Lindquist & Craig, 1988; Voos & Rottgers, 2002). This DnaJ homologue has been shown to have chaperone activity on its own (Voos & Rottgers, 2002).

Ecm10 shared an 82% amino acid homology with Ssc1 and 54% with Ssq1. It interacted with Mge1 in an ATP-

dependent manner and gene deletion did not lead to a change in phenotype (Baumann *et al.*, 2000).

Ssc1 and Ssq1 played sequential roles in the import and maturation of the yeast frataxin homologue (Voisine *et al.*, 2000). Deletion mutants of SSQ1 had a cold sensitive growth behaviour (Voos & Rottgers, 2002). Ssc1 carried out the functions of Ssq1, but this needed a 2000-fold excess over the normal levels of Ssq1.

### Hsp78

Hsp78 is a member of the hsp104 family found in the mitochondria (Moczko *et al.*, 1995) and is homologous to the C1p protein in *E. coli* (Leonhardt *et al.*, 1993). Cells with disrupted copies of hsp78 did not show a change in phenotype and hsp78 could substitute partially for the mitochondrial functions of hsp70 (Burnie & Matthews, 1991). The hsp78 refolding of luciferase was facilitated by Ssc1, indicating co-operation between hsp70 and hsp78 in mitochondrial protein folding (Krzewska *et al.*, 2001b). Purification of hsp78 demonstrated that it formed in an ATP-dependent manner, a homo-hexameric complex of approximately 440 kDa. Site-directed mutagenesis of the two putative nucleotide-binding domains (NBDs) suggested that the first was responsible for ATP hydrolysis and the second for protein oligomerization. The chaperone activity required not only Ssc1 but also Mdj1 and Mge1 (Krzewska *et al.*, 2001a). The thermosensitive restoration of mitochondrial DNA synthesis in *S. cerevisiae* required the reactivation of the mitochondrial DNA polymerase Mip1 through the action of both hsp70 and hsp78 (Germaniuk *et al.*, 2002). Hsp78 has also been implicated in the proteolysis required for the efficient degradation of substrate proteins in mitochondria (Rottgers *et al.*, 2002).

### Hsp90

*Saccharomyces cerevisiae* hsp90 was described originally in 1983 (Finkelstein & Strausberg, 1983). It constitutes 1–2% of all the protein in the eukaryotic cytosol and was essential for viability in yeasts, *Drosophila melanogaster* and man (Nathan *et al.*, 1997). In *S. cerevisiae*, there are two protein homologues named hsc82 and hsp82. Both have different patterns of expression, in that HSC82 is expressed constitutively at a very high level while HSP82 is induced more strongly by heat (Borkovich *et al.*, 1989). In living cells, large multidomain proteins have difficulty in reaching their native three-dimensional structures; they are prone to aggregate or become involved in kinetically trapped intermediates. Hsp90 is not perceived as being required for the *de novo* folding of most proteins, but it is required for a specific set of difficult-to-fold proteins. This includes three related tyrosine protein kinases, Wee1, Mik1

(*Schizosaccharomyces pombe*) and Swe1 (*S. cerevisiae*), along with Ste11, Gcn2 and the transcription factor Hap1. Swe1 was identified by the ability of the SWE1 gene to rescue mitotic catastrophe in a temperature-sensitive Wee1 mutant after expression in *S. pombe*. Swe1 phosphorylated Cdc28 and produced cell-cycle arrest when overexpressed (Goes & Martin, 2001). Ste11, the yeast equivalent of Raf in vertebrates, formed complexes with hsp90 and hsp90 was required for pheromone signalling in yeasts (Louvion *et al.*, 1998). Hsp90 enabled Ctf13 and Skp1 to nucleate the budding yeast kinetochore (Stemmann *et al.*, 2002).

The use of affinity chromatography with a fusion protein of histidine-tagged hsp82 from *S. cerevisiae* on a nickel-ion matrix demonstrated binding to Ssa, p60 (Sti1) and a 45-kDa immunophilin homologue (Chang & Lindquist, 1994).

The overexpression of candidal hsp90 (which has 84% identity to *S. cerevisiae*) in a laboratory strain of *S. cerevisiae* increased the yeast's virulence in mice, both in terms of mortality and colony counts in the kidney, liver and spleen (Hodgetts *et al.*, 1996).

Hsp90 was necessary for the assembly of human telomerase components. Mutations of STN1 which encode for Stn1, a protein involved in telomerase length regulation in yeasts, led to a lengthening of telomeric tracts. Overexpression of HSC82 slowed down this process, indicating a role of hsp90 in telomere maintenance (Grandin & Charbonneau, 2001).

Increased stress resistance extended the chronological life span of adult *Drosophila* and *Caenorhabditis elegans* by acting on postmitotic tissues. In yeasts, overactivation of the heat-shock response caused no slowing of replicative senescence but was associated with a longer life span in stationary cells (Harris *et al.*, 2001). Hsp90 has been implicated recently as central to the buffering effect, known as canalization (Stearns, 2003): the process whereby similar traits are produced in an organism despite genetic changes and environmental perturbation. Hsp90 has been described as suppressing phenotypic variation under normal conditions but releasing this variation when functionally compromised. This function was overwhelmed by environmental stress and exerted effects on key developmental processes (Bergman & Siegal, 2003).

Hsp90 inhibition by geldanamycin, radicicol, cisplatin and novobiocin accelerated cell lysis following either detergent or hypotonic shock. Cell lysis after hypoxia and complement attack was also enhanced by any form of hsp90 inhibition (Sreedhar *et al.*, 2003). Although hsp90 was highly conserved between eukaryotes, the expression of human *C. elegans* or *Candida albicans* sequences in yeasts strongly altered the sensitivity of the resulting recombinant strain to radicicol or geldanamycin (Piper *et al.*, 2003b). Hsp90 has been shown to have a critical role as a chaperone in the utilization of maltose as an alternative carbon source

where the client protein was MAL-activator, a DNA-binding transcription activator (Bali *et al.*, 2003).

Hsp90 has been found to complex with additional co-chaperones. The *trans/cis* isomerization of proline peptides has been shown to be a rate-limiting step in protein folding (Pirkel & Buchner, 2001). In *S. cerevisiae*, two homologues Cpr6 and Cpr7 complex with hsp90 via their carboxy-terminal tetratricopeptide repeats (TPRs) and have an amino-terminal peptidyl-prolyl *cis/trans* isomerase (PPIase) domain (Duina *et al.*, 1996b). Cpr6 was more stable than Cpr7 against thermal denaturation and displayed up to a 100-fold higher PPIase activity (Mayr *et al.*, 2000). Deletion of Cpr7 caused severe growth defects when combined with mutations that decreased the amount of hsp90 or Sti1 (Duina *et al.*, 1996a) and were hypersensitive to the hsp90 inhibitor geldanamycin (Dolinski *et al.*, 1998). These proteins have been described as the yeast homologues of mammalian cyclophilin 40. Overexpression of the tetratricopeptide domain of Cpr7 complemented Cpr7 mutant phenotypes, while overexpression of the cyclophilin domain of Cpr6, Cpr7 or human cyclophilin did not (Dolinski *et al.*, 1998). The growth suppression demonstrated in Cpr7 mutants was reversed by overexpression of *CNS1* which coded for *Cns1*, a Sti1/p60 homologue, which co-precipitated with hsp90 (Chang *et al.*, 1997; Marsh *et al.*, 1998).

Sti1 and Cpr6 both bind with submicromolar affinity to hsp90, Sti1 causing a large conformational change leading to a loss of ATPase activity (Prodromou *et al.*, 1999). These proteins act as a 'scaffold' for recruiting hsp70 and the target protein early in the process when ATP turnover was suppressed. Sti1 displaced geldanamycin. ATP suppression was also demonstrated by a second hsp90 co-chaperone, Cdc37p/p50, involved in the recruitment of tyrosine kinase clients. This co-chaperone formed a stable complex with geldanamycin-bound hsp90 (Siligardi *et al.*, 2002). Hsp90 and hsp70 have been shown to deliver preproteins to the mitochondrial import receptor Tom70. In its cytosolic segment, Tom70 contained seven TPR motifs. Deletion of Tom70 produced no change in phenotype because of partial functional overlap between Tom70 and Tom20 receptors (Steger *et al.*, 1990), while a double mutant was nonviable (Ramage *et al.*, 1993). In yeasts, unlike mammals, hsp70 rather than hsp90 is used to import preproteins into the mitochondria and hsp70 docking is required for the formation of a productive protein-Tom70 complex (Young *et al.*, 2003).

In mammalian cells, the interaction of hsp90 with steroid hormone receptors requires the co-operation of p23. The yeast equivalent has been designated Sba1. Deletion of the gene SBA1 did not prevent the interaction of hsp90 with its target proteins but it increased sensitivity to ansamycin antibiotics (Bohen, 1998). Expression in the fusion yeast of Sba1 and Wos2 demonstrated that they were functionally

exchangeable. Wos2 was abundant in the yeast cell and expression dropped dramatically when the yeast entered the stationary phase (Munoz *et al.*, 1999). Sba1 was functionally indistinguishable from human p23 in seven assays involving members of the intracellular receptor family (Freeman *et al.*, 2000). The dioxin receptor has been described as an hsp90-regulated transcription factor which bound to 2,3,7,8-tetrachlorodibenzodioxin. In a yeast model system, deletion of SBA1 reduced ligand-mediated dioxin receptor signalling by approximately 40% (Cox & Miller, 2002).

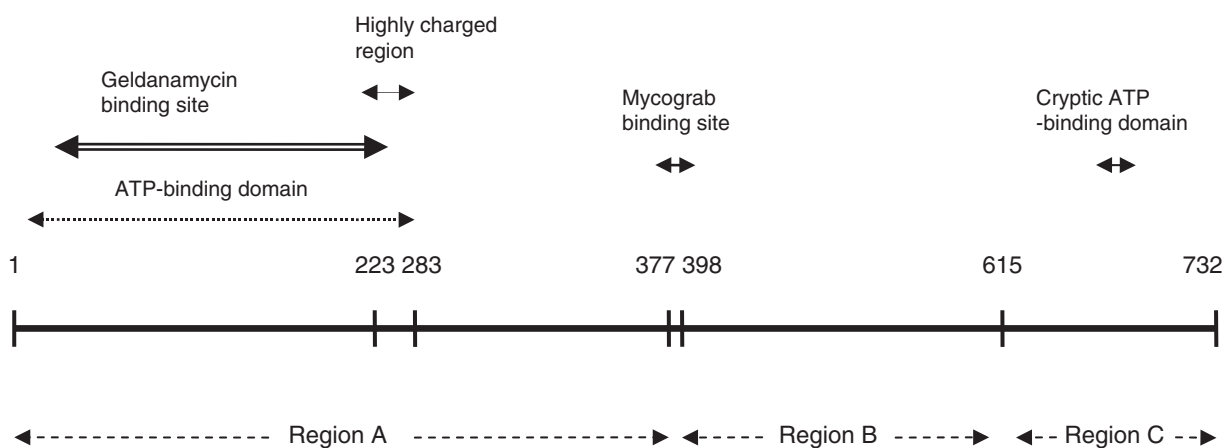
The intrinsic ATPase activity of hsp90 was enhanced by Aha1 and suppressed by Hch1. Aha1 and Hch1 were crucial for cell viability under nonoptimal growth when hsp90 levels were limiting (Lotz *et al.*, 2003). In eukaryotic cells, proteins for degradation were degraded by the 26S proteasome after covalent attachment to ubiquitin. In yeasts, the functional loss of hsp90 in a temperature sensitive mutant is associated with dissociation of the 26S proteasome. Hsp90 plays a principal role in the assembly and maintenance of this 26S proteasome.

The primary structure of hsp90 has been subdivided into three regions. These are the amino end Region A (residues 1–400), middle Region B (residues 401–615) and carboxy end Region C (residues 621–732) (Matsumoto *et al.*, 2002). The amino end has the ATP- and geldanamycin-binding domains, and has been subdivided into the ATP-binding domain (residues 1–283) and the highly charged region (residues 223–283) (Fig. 1). The latter has been suggested as being exposed on the outer surface of the molecule and appeared to be dispensable for viability and signal transduction (Louvion *et al.*, 1996). It may have a role in modulating the function of the ATP-binding site and the binding of p23 (Scheibel *et al.*, 1999). The structure of the ATP-binding site made it a member of the GHKL family with a type of fold

similar to DNA gyrase B and Mut1 (Dragon *et al.*, 1985; Frydman, 2001). The adenosine base and ribose were buried in a kinked conformation in a protein cleft. Binding of HTP led to a conformational change in hsp90 and the trapped ATP was committed to hydrolysis. Heterodimers between the wild-type protein and a mutant that lacked ATPase activity still had the ability to stimulate ATPase activity in the wild-type molecule (Richter *et al.*, 2001). This activity was dissected further to the first 24 amino acids (Richter *et al.*, 2002), which acted as an 'ATP lid' (Dehner *et al.*, 2003).

Using a yeast twofold screen the 12-kDa carboxy-terminal domain of human hsp90 $\alpha$  has been shown to mediate the interaction with TPR-containing sequences in cofactors such as FKBP51/54 and FKBP52, p60 and Cyp40 (Young *et al.*, 1998). Peptide binding to hsp90 has been localized to the EEVD motif with the carboxy-terminal aspartate acting as a two-carboxylate anchor (Scheufler *et al.*, 2000). Carboxy-terminal truncated mutants lacked the inherent ability to dimerize and displayed a reduced ATPase activity. They dimerized in the presence of 5'-adenyl-amino-diphosphate. This molecule was also required for the recruitment of Sba1 (Prodromou *et al.*, 2000). The carboxy-terminal (residues 333–664) contained the sequence responsible for the cytoplasmic location of the protein in humans (Passinen *et al.*, 2001). Sti1 binding was absent in a mutant lacking the last five amino acids, MEEVD (Richter *et al.*, 2003).

A second ATP-binding site has been demonstrated in the carboxy end. Secondary structure prediction revealed motifs compatible with a Rossmann fold, with peptide residues 657–677 crucial for nucleotide and novobiocin binding (Garnier *et al.*, 2002). This cryptic carboxy-terminal site opened only after occupancy of the amino-terminal site (Soti *et al.*, 2002). The carboxy-terminal-binding site, unlike



**Fig. 1.** Hsp90 of *Saccharomyces cerevisiae*. Schematic representation of the characterized regions of hsp90 from *S. cerevisiae*, including the Geldanamycin and Mycograb<sup>®</sup> binding sites (see section on 'hsp90' in this section). The numbers represent the amino acids and the arrows represent the location of the binding sites.

the amino-end-binding site, interacted with both purine and pyrimidine nucleotides (Soti *et al.*, 2003).

The middle domain associated simultaneously with the amino and carboxy ends. The involvement of the carboxy end was abolished by replacement of either leucine within the hydrophobic segment (residues 662–678) (Yamada *et al.*, 2003). Introduction of a cysteine residue at the carboxy but not the amino end resulted in a molecule which was able to trap ATP as efficiently as the parent molecule (Wegele *et al.*, 2003). Aha1 and Hch1 have been shown to bind to this region and this resulted in a fivefold rise in the ATPase activity of hsp90. Impaired activation of the protein v-Src and reduced cell viability were seen when the genes AHA1 and HCH1 were deleted. In GHKL protein family members, this middle segment has been perceived as sensing the presence of the *g*-phosphate of ATP and orientating it for attack by a nucleophilic water (Meyer *et al.*, 2003). In hsp90, the topologically equivalent loop contained the highly conserved motif 377-N (L/I/V) SRE X LQ-384, in which the conserved N, R, Q provided the *g*-phosphate interaction. Mutating either the arginine or glycine produced strains incapable of growth at any temperature.

### Hsp104

HSP104 is a member of the highly conserved HSP100 gene family. In *E. coli*, this consists of the three subfamilies ClpA, ClpB, ClpC and the nearest homologue to hsp104 is ClpA (Parsell *et al.*, 1994). Its expression was crucial in the survival of stationary-phase yeast cells and it governed their stability at high temperatures, tolerance to ethanol and radiation and moderate tolerance to sodium arsenite (Sanchez *et al.*, 1992; Boreham & Mitchel, 1994). There was similarity between the functions of hsp70 and hsp104. In the absence of hsp104, hsp70 was important in maintaining thermotolerance (Sanchez *et al.*, 1993).

Hsp104 has two NBDs (NBD1 and NBD2). Mutation in NBD1 reduced ATPase activity, while mutation in NBD2 reduced the ability of hsp104 to oligomerize into ring-shaped hexamers (Costantino *et al.*, 1994; Corrochano, 2002). Hsp104 did not prevent the aggregation of denatured proteins, but in concert with hsp40 and hsp70 can reactivate proteins that have been denatured and allowed to aggregate (Glover & Lindquist, 1998).

NBD1 and NBD2 had different catalytic activities and each displayed cooperative kinetics for ATP hydrolysis. The effect of mutation on the loss of function of hsp104 in thermotolerance and yeast propagation assays demonstrated that ATP hydrolysis was a distinct process from ATP binding (Hattendorf & Lindquist, 2002).

The level of active hsp104 correlated with the propagation of the yeast (PSI<sup>+</sup>) prion elements in that both the over- and underexpression of hsp104 cured yeast cells. Guanidine

hydrochloride cured yeast cells by inhibiting hsp104 activity (Jung & Masison, 2001). The latter prion consisted of the aggregation-prone isoform of the translational termination factor Sup35 (eRF3). Hsp104 was also involved in the propagation of the prions (PIN<sup>+</sup>) and (URE3) and has been suggested as playing a role in the replication of all yeast prions (Wegrzyn *et al.*, 2001). The amino acid residue 184 of hsp104 was critical to prion-curing by guanidine, prion propagation and thermotolerance (Schirmer & Lindquist, 1997).

When Sup35 and hsp104 were mixed, the circular dichroism spectrum differed from that predicted by the addition of the proteins' individual spectra and the ATPase activity of hsp104 was inhibited. This supported a direct interaction of the two molecules coupled to a change in structure of hsp104, leading to a loss of ATPase activity (Schirmer & Lindquist, 1997).

Control of the expression of HSP104 has been delineated to a 334-bp fragment proximal to the first coding AUG (Grably *et al.*, 2002). Within this, a 260-bp fragment was essential for the heat-shock response. This contained both HSEs and STREs which, as with hsp26, need to cooperate to produce maximal inducible expression. The contribution of Msn2p and Msn4p for hsp26 and hsp104 was gene- and stress condition-specific (Amoros & Estruch, 2001). Hsp104 interacted with the co-chaperones Sti1, Cpr7 and Cns1, which utilize the TPR domains. This interaction was hsp90-independent, suggesting that it was a dual response to altered metabolic conditions (Abbas-Terki *et al.*, 2001).

### Trehalose and hsp

*Saccharomyces cerevisiae* cells grown at 24 °C require pre-conditioning by growing at 37 °C before they acquire tolerance to brief exposure at 48–50 °C (Simola *et al.*, 2000). This is associated with an increase in cytoplasmic trehalose. In these extreme stress conditions hsp104 was needed to rescue the heat-inactivated proteins from insoluble aggregates. Hsp104 produced the disaggregated protein, which acted as substrate for folding by hsp70 and hsp40 (Estruch, 2000). During heat shock, hsp104 contributed to both the simultaneous accumulation and degradation of trehalose (Iwahashi *et al.*, 1998). Trehalose facilitated the refolding of glycoproteins in the endoplasmic reticulum after severe heat stress. This process failed in a mutant where the TPS1 gene, coding a subunit of trehalose synthase, was absent (Simola *et al.*, 2000). Trehalose has also been recognized as a key molecule in stabilizing proteins and biological membranes in yeasts in response to desiccation, barotolerance (resistance to hydrostatic pressure), nutrient starvation, osmotic or oxidative stress and exposure to toxic chemicals. The contribution of hsp104 to barotolerance was temperature-dependent, whereas that of trehalose was



not (Iwahashi *et al.*, 1997; Voit, 2003). A sterol auxotrophic mutant of *S. cerevisiae* demonstrated that yeast cells with higher levels of sterol had a greater tolerance to heat and ethanol stress. This was independent of any effect on trehalose and hsp104 (Swan & Watson, 1998). The proteasome inhibitor MG132 had little effect on the growth rate of *S. cerevisiae* but caused a dramatic increase in resistance to high temperature. When MG132 was removed this thermotolerance decreased rapidly while, paradoxically, the synthesis of hsp's continued to increase. This effect was linked to the accumulation of trehalose, as MG132 and previous exposure to 37 °C had additive effects on trehalose accumulation (Lee & Goldberg, 1998).

Cellular responses to stress are regulated in concert with metabolic oscillation (Saito *et al.*, 2002). This coupling was inactivated by disruption of the GTS1 gene. The product Gts1 played an important role in the regulation of heat tolerance in yeasts grown in glucose-limited conditions. The disruption of SNF1, a transcriptional activator of glucose-repressible genes, diminished the effect of GTS1. Mutations in GTS1 altered the intracellular levels of hsp104 and trehalose in proportion to the gene dosage of GTS1. A variant of GTS1 lacking the Q-rich domain lost its ability to activate HSP104 and TGS1. This suggested that GTS1 increased heat tolerance in the stationary phase of growth by activating a Snf1 kinase-dependent depression of HSP104 and TPS1 (Yaguchi & Tsurugi, 2003).

## Fermentation

During the process of industrial fermentation, yeast cells are exposed to complex and varied carbon sources and stressed by anaerobic growth, high specific gravity, ethanol, temperature shocks and storage for extended periods at a low temperature. This elicits a variety of stress responses, which are often strain-specific. For example, the maximum expression of hsp104 occurred in top fermenters when exposed to 41 °C, while in bottom fermenters this occurred at 37 °C (Brosnan *et al.*, 2000). Hsp induction was linked to a stress response to ethanol in wine-producing strains, but the level of expression was strain-specific (Ivorra *et al.*, 1999). In sherry strains, a point mutation in HSP12 or deletion of the entire gene prevented the production of a biofilm at the end of ethanol fermentation (Zara *et al.*, 2002). Hsp104, hsp78, hsp30 and hsp26 were expressed during wine fermentation, with the dominant molecules being hsp26 and hsp30. Hsp30 was induced during the late stationary phase and remained active until the end of the wine fermentation (Riou *et al.*, 1997).

## HSF

Yeast HSF has been shown to be highly conserved with its mammalian homologue. It is activated by multiple stresses, including heat, oxidative stress, glucose starvation, pH and

salicylate (Hahn & Thiele, 2004). Approximately 165 *in vivo* targets for HSF have been identified by chromatin immunoprecipitation and DNA microarray. These include most of the hsp's where HSF binds to the HSEs present in their promoters (Zarzov *et al.*, 1997). Each HSE contains two or more contiguous inverted repeats of the 5-bp sequence nGAAn. The HSF gene was essential even in the absence of heat stress. Human HSF2, but not HSF1, rescued yeast cells lacking HSF and this function correlated with its ability to form homotrimers. HSF2 also allowed the yeast cells to acquire thermotolerance (Liu *et al.*, 1997). Yeast HSF can exist in two conformations, an unstressed 'low-activity' conformation and a stressed 'high-activity' conformation. This was associated with a change in electrophoretic mobility and required two HSF trimers to bind to the DNA (Lee *et al.*, 2000).

Yeast HSF is under negative regulatory control in the absence of stress. This has led to a model where in the basal state HSF has been tied up with 'free' hsp's in a regulatory loop. This has been challenged by the observation that overexpression of Ssa2, a prominent candidate molecule in this feedback regulation, did not inhibit the heat-shock response (Hjorth-Sorensen *et al.*, 2001). This led to a concept where HSF was activated by the direct sensing of heat or superoxide, which triggered a conformational change. The molecule was then refolded with the assistance of the Ssa proteins. Ssb proteins were perceived as fine-tuning the process (Bonner *et al.*, 2000).

Yeast HSF has been described as having several clearly defined domains (Bulman *et al.*, 2001). There was a central core responsible for DNA binding and oligomerization, a trimerization domain, a DNA-binding domain and two activation domains located at the amino and carboxy termini. Deletion of the carboxy activation domain resulted in a yeast which could not grow at higher temperatures. This domain was critical for the increase of expression of the metallothionein gene (CUP1), in response to glucose starvation (Bulman *et al.*, 2001). Mutations in a conserved arginine (residue 274) at the carboxy-terminal end of the DNA-binding domain increased both the basal level of activity of HSF at normal temperatures and the induced activity on heat shock (Chen & Parker, 2002).

HSF has been shown to interact with HSP90. Reduction in levels of hsp90 activated the molecule, a phenomenon which was mimicked by the addition of geldanamycin (Zou *et al.*, 1998). HSF binds to the HSEs in the variant DNaseI-hypersensitive domain localized at the 5' end of HSP90. HSE mutations abolished the transcription and *in vivo* occupancy of the nearby TATA box (Erkine *et al.*, 1996).

## *Neurospora crassa*

In *N. crassa*, asexual development can be divided into four stages, starting with dormant conidia which germinate

within a few hours on suspension in water. This then leads to vegetative hyphae, which grow and branch to form a mycelial mat. These produce aerial hyphae forming conidia.

In initial studies with *N. crassa*, cycloheximide inhibited about 70% of protein synthesis and upregulated three proteins of 28, 30 and 58 kDa. Heat shock from 25 to 37 °C induced a 70-kDa protein (Perlman & Feldman, 1982). At elevated temperatures, germinating conidiospores initiated major hsps at 67, 83 and 98 kDa and a minor hsp at 30 kDa. The response was maximum at 45 °C and a variation of 1 °C produced a large difference in the amount and pattern of proteins produced (Plesofsky-Vig & Brambl, 1985). Both conidiospores and mycelial cells resumed normal protein synthesis after 60 min at high temperature (Plesofsky-Vig & Brambl, 1987). Transfer of mycelium to a medium supplemented with ethanol stimulated production of an 80-kDa hsp even at 28 °C (Roychowdhury & Kapoor, 1988). This molecule was purified and in the native state shown to have an apparent molecular mass of 610 kDa (Roychowdhury & Kapoor, 1990). A polyclonal serum against it was used to screen an expression library in  $\lambda$ gt11. Analysis of a partial nucleotide sequence from a positive clone demonstrated 85% identity of the amino acid sequence with yeast hsp90. Expression was demonstrated by Northern blotting of the mycelium produced in response to heat shock (Roychowdhury *et al.*, 1992). Hsp80 was shown to bind to hsp70 and had the ability to interact with an unfolded polypeptide individually or in a complex (Freitag *et al.*, 1997; Ouimet & Kapoor, 1998). Subsequently, this hsp90 homologue was shown to be expressed in dormant conidia (Girvitz *et al.*, 2000).

The 98-kDa hsp was identified subsequently as having a 71% homology to the amino acid sequence of hsp104 from *S. cerevisiae* (Vassilev *et al.*, 1992). The 30-kDa hsp was shown to be related to the  $\alpha$  crystalline-related hsps. It was present as a single copy and hsp30-defective strains were extremely sensitive to the combined stresses of high temperature and carbohydrate limitation (Plesofsky-Vig & Brambl, 1995). There was a 35% reduction in hexokinase activity (Plesofsky & Brambl, 1999) and the import of selected proteins into the internal compartments of the mitochondria was inhibited (Plesofsky *et al.*, 1999). The conserved  $\alpha$ -crystalline domain could be divided into N- and C-terminal subdomains which interacted with each other (Plesofsky & Brambl, 2002). Hsp30 was shown to bind to hsp70 and hsp88 (Plesofsky-Vig & Brambl, 1998).

The gene coding the major 70-kDa hsp has been cloned. The coding region was found to contain four introns with boundaries and internal consensus motifs (Kapoor *et al.*, 1995). Immunoblot and enzyme-linked immunosorbent assay (ELISA) analyses demonstrated the highest levels of hsp70 in aerial and dormant conidia. During germination the level fell, but this was reversed during vegetative growth (Fracella *et al.*, 1997). A second hsp70 homologue of 72 kDa

was identified as the gene encoding the glucose-regulated protein grp78. The gene had five introns and the endoplasmic retention signal HDEL at its carboxy terminus. Expression was constitutive but enhanced by starvation, treatment with tunicamycin or growth at 40 °C (Techel *et al.*, 1998). The expression increased during germination and exponential growth, declined in young aerial hyphae and reached a maximum in late aerial hyphae. This late production was related to developmental activation of transcription or a lower rate of mRNA degradation during this stage (Hafker *et al.*, 1998).

The HSF of *N. crassa* has been shown to bind constitutively to HSEs under nonstress conditions while heat shock induced an alteration in HSF which led to its fast inactivation (Meyer *et al.*, 2000; Monnerjahn *et al.*, 2001).

### ***Achlya ambisexualis***

*Achlya ambisexualis* is an oomycete fungus where sexual reproduction results from the co-operation of two strains of different mating types. This is controlled by antheridiol released from the female strains and oogonia from the male strains. In the male strain, antheridiol induced specialized structures termed antheridial branches on the fungal hyphae which differentiated into antheridia. The male hyphae then secreted oogoniol, which caused the female strain to develop oogonia. The antheridial branches then grew towards the oogonia which produced fertilization (Brunt & Silver, 1986). An HSP90 homologue was upregulated in response to both heat shock and antheridiol (Brunt *et al.*, 1990). Molecular cloning demonstrated two homologues, of which the expression of one was controlled by antheridiol (Brunt & Silver, 1991), as was the expression of three different hsp70 homologues (Brunt *et al.*, 1998a). The two hsp90 homologues of 84 and 86 kDa can be distinguished in immunoblots of mycelia proteins, as the one at 86 kDa reacts with an antiphosphoserine antibody (Brunt & Silver, 2004). Co-precipitation of cytosolic proteins with *Achlya* hsp90 identified proteins of 23, 27, 47, 56, 61, 74 and 110 kDa. The 74-kDa protein was an hsp70 homologue, the 56-kDa protein related to immunophilin FKBP51 and the 23 kDa related to the vertebrate protein p23. The 27-, 61- and 110-kDa proteins appeared unique to the *Achlya* complexes (Brunt *et al.*, 1998b).

## **Hsps and pathogenic fungi**

### ***Candida albicans***

Initial studies (Dabrowa & Howard, 1984; Zeuthen *et al.*, 1988; Zeuthen & Howard, 1989; Dabrowa *et al.*, 1990) examined the expression of cytoplasmic proteins in response to heat shock, ethanol, nutritional stress and thermo-

tolerance followed by heat shock. Heat shock from 23 to 37 °C induced proteins at 18, 22, 40, 68 and 70 kDa (Dabrowa & Howard, 1984). The proteins at 40, 68 and 70 kDa were undetectable on gels when cells were incubated at 23 °C, appeared within 20 min of the temperature shift, and were no longer seen after incubation at 37 °C for 2 h. The 18- and 22-kDa proteins were present at 23 °C, the concentrations increased greatly and persisted after temperature shift. At 45 °C (Zeuthen & Howard, 1989), nine hsp's at 18, 26, 34, 54, 72, 76, 81, 85 and 98 kDa were visualized. In *Candida psychrophila*, which being a psychrotroph grew at 0 and 20 °C, hsp's were induced at 25 °C which were absent from cells grown at 15 °C. Yeasts grown at 25 °C acquired tolerance to 37 °C and 100 mM hydrogen peroxide. Hsp's were induced at 80 and 110 kDa, whereas hsp's at 60, 70 and 90 kDa were expressed throughout (Deegenars & Watson, 1997). HSP12 was upregulated in a mutant of *C. albicans* SSK1, which was sensitive to several oxidants including hydrogen peroxide. Ssk1 was the putative regulator protein of the Hog1 two-component signal transduction system. This regulated the mitogen-activated protein (MAP) kinase two-component signal pathway in *S. cerevisiae* when cells were confronted with osmotic conditions (Chauhan *et al.*, 2003). Other hsp's have been identified by growing *C. albicans* at 42 °C. Induced bands on electrophoresis were visible at 28, 38, 47 and 60 kDa, although the predominant response was in the 70–110-kDa range (Franklyn & Warmington, 1994). Two-dimensional electrophoresis of extracts of *C. albicans* identified homologues of *S. cerevisiae* hsp90, Ssa1, Ssa4, Ssb1/Kar2, Ss31/Pdr13 and hsp104 (Pitarch *et al.*, 2002).

Antibodies to a mycobacterial 65-kDa stress protein have been shown to be elevated in patients with chronic atrophic oral candidiasis, vulvo-vaginal candidiasis, oral lichen plans and recurrent aphthous ulceration, when compared with subjects with clinically healthy oral mucosa (Ivanyi & Ivanyi, 1990). In HIV infection, raised serum and salivary IgA antibodies were shown against mycobacterial hsp65 and concurrent *Candida* infection was proposed as modifying both the antibody titre and relative avidity (Coogan & Challacombe, 2000). Antibodies to hsp60 and hsp70 were raised in vaginal wash samples in women with recurrent vulvo-vaginitis (Giraldo *et al.*, 1999a,c). The occurrence of antibody against hsp70 was significantly higher in women with bacterial vaginosis or yeast infection (Giraldo *et al.*, 1999b). In chronic infections, the presence of secondary antigens becomes of increased significance. Increased levels of antibodies to hsp70 indicate that hsp70 may be an important secondary immunogenic factor.

### Stress mannoproteins

Mannoproteins with molecular masses of 60–70, 90–110, 130–150 and 180–200 kDa were produced by *C. albicans*

when isolates grown in a synthetic medium were heat-shocked from 25 to 37 °C. These reacted with the secretory IgA from patients with oral and vaginal candidiasis (Polonelli *et al.*, 1994). The major glycoprotein had a molecular mass of 200 kDa and expression was reduced when clinical isolates of *C. albicans* were subcultured from the mucosal surface of a patient onto Sabouraud's dextrose agar (Ponton *et al.*, 1996). The expression and secretory activity of tumour necrosis factor in the murine macrophage cell line ANA-1 was induced in a dose- and time-dependent manner by this mannoprotein (Pitzurra *et al.*, 1996). The expression of these stress mannoproteins also required optimal concentrations of glucose and ammonium sulphate in the growth medium. Mannoproteins were found in all 10 *C. albicans* isolates studied and were also found in nine of 28 non-*C. albicans* isolates, indicating that these proteins are not specific to *C. albicans* (Vidotto *et al.*, 1998).

### Hsp70

Screening an expression library of the yeast form of *C. albicans* with rabbit polyclonal antibodies against heat-activated *C. albicans* cells identified two clones encoding hsp70. The derived sequence was 84% similar to the SSA1 gene of *S. cerevisiae*. Most of the nonconservative substitutions were between amino acids 557–644, which was perceived as an area key to the antigenicity of the molecule (La Valle *et al.*, 1995). Immunization with a recombinant of this molecule produced a murine antibody response against bands at 70 kDa, which were suggested as similar to a 75-kDa hsp shown previously as immunodominant in CBA/H mice during systemic candidiasis (Costantino *et al.*, 1994). Immunoscreening a mycelial expression library further identified a sequence which was 99.3% identical to *C. albicans* HSP70 and approximately 79% identical to the sequences of SSA1–4 (Eroles *et al.*, 1995).

A further hsp70 homologue was cloned after screening a cDNA expression library with rabbit antiserum against a cell wall extract (Alloush *et al.*, 1996). Expression of this protein was characterized by immunofluorescence with an affinity-purified antibody against recombinant hsp70 to the cell surface of the yeast. The sequence of this gene was most similar to SSA2 and the gene was designated *C. albicans* SSA2. Northern blot analysis demonstrated gene expression at both 25 and 37 °C and during germ tube formation in Lee medium at 37 °C. The recombinant variant reacted with the sera of healthy individuals and patients with candidiasis (Lopez-Ribot *et al.*, 1996). A further HSP70 gene (SSB1) has been cloned from *C. albicans* with 85% similarity to the SSB subfamily of *S. cerevisiae* (Maneu *et al.*, 1997). Northern blot analysis confirmed that SSB mRNA levels increased after a mild cold shift (28–23 °C) and decreased rapidly after a mild heat shift (from 28 to 37 °C) (Maneu *et al.*, 2000). CaMsi3p,

a putative member of the hsp70 family with 63% homology to Msi3p/Sse1p from *S. cerevisiae*, has also been identified. It was approximately 28% identical to the Ssa1 and Ssb1 homologues of *C. albicans*. CaMSI3 complemented a temperature-sensitive Msi3 mutant of *S. cerevisiae* (Cho *et al.*, 2003).

Sequences derived from candidal hsp70 (La Valle *et al.*, 1995) have been used in a polymerase chain reaction (PCR) to differentiate between *C. albicans* and other yeast species. The sensitivity of this assay was as low as 10 fungal cells (Arancia *et al.*, 1997). Control of hsp70 expression (La Valle *et al.*, 1995) is by three HSEs and one STRE that bind to candidal HSF (Sandini *et al.*, 2002). Immunization of mice with a recombinant form of hsp70 confirmed the antigenicity of hsp70, especially the 21-kDa carboxy-terminal fragment. Mice immunized with either the complete recombinant protein or the amino end demonstrated an enhanced susceptibility in a lethal mouse model of infection. There was rapid production of interleukin-6 and tumour necrosis factor- $\alpha$  (Bromuro *et al.*, 1998). This was in contrast to other work, which suggested that both enolase and hsp70 were present in the cell walls of *C. albicans* and acted as a 'smokescreen' that subverted an effective host immune response to critical surface epitopes on fungal antigens. Here antibody against hsp70 would be perceived as beneficial, because it would prevent the yeast evading the host's immune system (Eroles *et al.*, 1997).

Ssa1/2 has been described recently as the fungal cell envelope protein that binds to receptors for histatin 5. This was the most potent of the 12 histatin family members, with fungicidal activity at physiological concentrations in saliva. The mechanism involved binding with Ssa1/2, followed by the intracellular translocation and efflux of potassium, magnesium and ATP (Li *et al.*, 2003).

## Hsp90

Hsp90 was the first hsp to be sequenced from *C. albicans*. The sequence was derived from a clone identified by screening a genomic library made in the expression vector lambda gt11 with antibody from patients who had recovered from disseminated candidiasis. This pulled out the carboxy-end 394 amino acids, which were then expressed in a lysogen under the control of the *lacZ* promoter in *E. coli* Y1089. The resultant 160-kDa fusion protein reacted with rabbit candidal antiserum, sera from AIDS patients with antibody to a *Candida* antigen of 47 kDa, but not with sera from HIV antibody-positive patients without evidence of this antibody on an immunoblot. A patient with disseminated candidiasis who seroconverted to the 47-kDa band also seroconverted to this fusion protein (Matthews & Burnie, 1989).

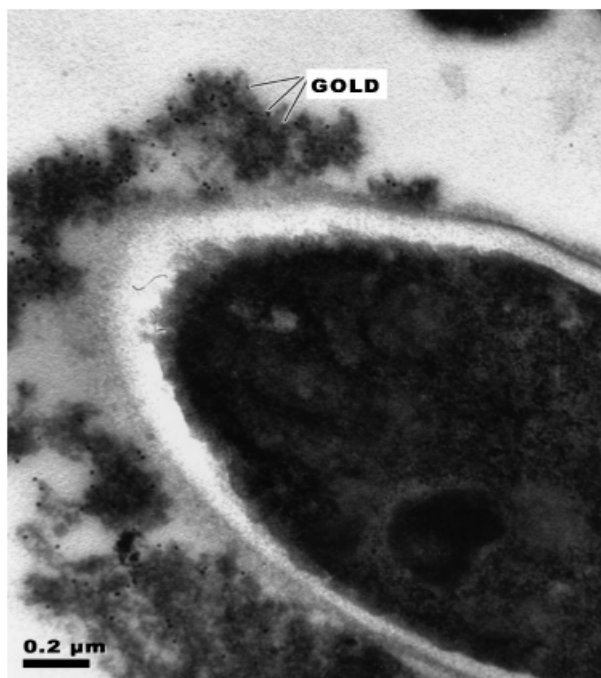
Identical full sequences were obtained by sequencing clones obtained from genomic libraries of *C. albicans* 3153A and ATCC 10261. Northern analysis demonstrated a

ninefold increase in mRNA levels when the yeast was subjected to a temperature shift from 25 to 45 °C. Southern blotting confirmed the existence of a single locus in *C. albicans* in contrast to the closely related genes HSC82 and HSP82 in *S. cerevisiae*. Hsp90 mRNA levels increased transiently during yeast to hyphal transition, but this did not correlate with the production of germ tubes. Attempts to produce a null mutant were unsuccessful (Swoboda *et al.*, 1995a, b).

A 92-kDa protein, which was inducible by heat shock and oestrogen in *C. albicans*, was identified by direct amino acid sequencing as a cobalamin-independent methionine synthase (Burt *et al.*, 1999).

The creation of a construct in *S. cerevisiae* (using the *C. albicans* hsp90 sequence to replace the *S. cerevisiae* hsp90 sequence) demonstrated that the *Candida* sequence could replace the *Saccharomyces* sequence and maintain viability (Panaretou *et al.*, 1999). Growth of three *C. albicans* strains in the presence of 17- $\beta$ -oestradiol demonstrated that intracellular hsp90 was upregulated. The results were confirmed by reverse transcriptase PCR with peak expression after a 2-h exposure (Zhang *et al.*, 2000). *Candida albicans* does not have a true oestrogen receptor or oestrogen response elements homologous to those found in man (Malloy *et al.*, 1993). Oestradiol in some strains of *C. albicans* induced larger colonies and germ tube formation in activated-charcoal-stripped serum. The tyrosine kinase inhibitor, genistein, also stimulated growth, whereas the oestrogen antagonist, nafoxidine, reduced it (Yazdanyar *et al.*, 2001). Oestradiol also upregulated CDR1 (Zhang *et al.*, 2000), which coded for an ABC transporter gene responsible for fluconazole resistance (White *et al.*, 2002). In *S. cerevisiae*, cells were rendered hypersensitive to hsp90 inhibitors by mutation in hsp90 itself and through loss of the specific plasma-membrane ABC transporters, Pdr5p and Snq2p (Piper *et al.*, 2003a). Thus, elements which tend to regulate hsp90 also tend to regulate CDR1 and therefore fluconazole resistance.

Similarly, homologues to Aha1 and Hch1 have been identified in *S. cerevisiae* (Lotz *et al.*, 2003). An hsp90 homologue has been cloned from *Candida tropicalis* using a PCR-based approach (Santhanam & Burnie, 2000). Recently the ability of *C. albicans* and *Candida glabrata* to undergo sexual mating has been described (Panwar *et al.*, 2003; Wong *et al.*, 2003). The mating pheromone of *C. albicans* has been shown to induce approximately 62 genes. These coded for proteins from the cell surface and secreted proteins implicated previously in the virulence of *C. albicans* in a mouse model of disseminated candidiasis (Bennett *et al.*, 2003). Paradoxically, no hsps were upregulated, despite the involvement of hsp90 in the response of *S. cerevisiae* to pheromone (Stemmann *et al.*, 2002) and the upregulation of a conserved MAP kinase (Chen *et al.*, 2002), which is likely to be a target molecule for hsp90 folding.

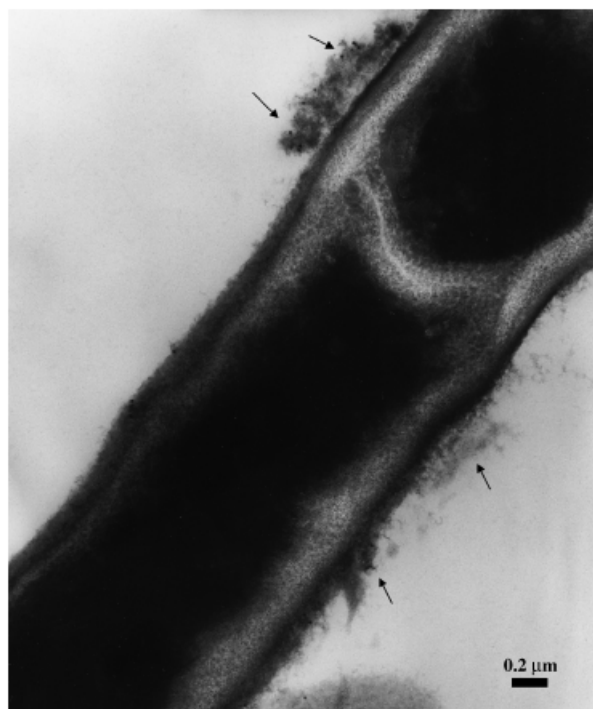


**Fig. 2.** Hsp90 located on the cell wall of *Candida albicans* using pre-embedded immunolabelling. Immunogold electron micrographs showing hsp90 production on the cell surface indicated by the concentration of gold particles (the black dots indicated by the lines) on the cell wall of a yeast (*C. albicans* grown at 37 °C).

Immunoelectron microscopy confirmed that this protein is a component of the cell wall of *C. albicans* and showed that hsp90 is located on the cell wall surface. The protein was found in different concentrations among the cells, which indicated that the expression of cell wall components is a dynamic process influenced by environmental and nutritional conditions (Fig. 2). The presence of large amounts of the hsp90 in the tips of the hyphae is an interesting finding (Fig. 3). It has been demonstrated that hyphae are associated with virulence in *C. albicans*. Furthermore, the tips of the hyphae have been reported to have roles in pathogenesis and virulence. The hyphal tip is also the site of secretion of enzymes, which have the ability to breakdown proteins, lipids and other cellular components (Hube & Naglik, 2001). Therefore, hsp90 may play a role in the pathogenesis and virulence of *C. albicans* (Albarrag, 2004).

### ***Cryptococcus neoformans***

Initial studies in murine pulmonary *Cryptococcus* identified a 77-kDa protein as immunogenic. This cross-reacted with a rabbit polyclonal serum against hsp70 and was confirmed as a member of the hsp70 protein family by direct amino acid sequencing. A second nonimmunogenic 66-kDa protein was identified as a member of the hsp60 family of proteins



**Fig. 3.** Hsp90 located on the cell wall of *Candida albicans* hypha and congregated at the hyphal tips using pre-embedded immunolabelling. Immunogold electron micrographs showing *C. albicans* hypha with gold particles on the surface, indicating hsp90 production (the black dots indicated by the arrows) on the cell wall of a yeast (*C. albicans* grown at 37 °C).

(Takeya *et al.*, 1997). This study was extended to human pulmonary infection, where approximately two-thirds of patients produced a humoral response to this protein and its 43-kDa breakdown product. This response was predominantly to sequences at the carboxy end (Takeya *et al.*, 1999). The ability of *C. neoformans* to grow at an elevated temperature was essential for virulence and was reversed in a mutant with a defective MAP kinase (Kraus *et al.*, 2003). Serial analysis of gene expression (SAGE) of *C. neoformans* cells, recovered from the central nervous system of infected rabbits, has been performed. This demonstrated the importance of hsp12, hsp60, hsp70 and hsp90, as judged by their expression during the process of infection (Steen *et al.*, 2003). A pheromone-dependent fungal mating system has also been shown for *C. neoformans* (Chaturvedi *et al.*, 2002). This, as for *S. cerevisiae* (Louvion *et al.*, 1998), may need hsp90 to function properly. Upon ligand binding, the hormone-binding domain underwent a conformational change that resulted in the release of hsp90 and the concomitant activation of the steroid receptor. Hsp90 may have a dual role: it ensures that receptors are kept inactive in the absence of hormone, and helps them to respond specifically and efficiently to ligand. We speculate that in

*C. neoformans* hsp90 interacts with the Ras1 signal transduction pathway which controls mating, hyphal differentiation and the ability to grow at elevated temperatures. Ras1 is required for the transcriptional induction of elements of the pheromone response pathway by activating the MAP kinase cascade and regulation of cAMP production. Although *C. neoformans* Ras1 mutant strains are viable they are unable to grow at 37 °C, are avirulent in rabbit and mice models and are unable to undergo hyphal transition during mating. Louvion *et al.* (1998) demonstrated that pheromone signalling through the MAP kinase cascade depends on hsp90 function.

### ***Aspergillus* spp.**

In *Aspergillus nidulans*, cDNA coding for a protein with 42% sequence homology to *N. crassa* hsp90 has been identified. Expression was high at a normal temperature and was only slightly elevated by heat shock (Kusakabe *et al.*, 1994). Trehalose has been identified as a stress metabolite and the gene encoding trehalose-6-phosphate synthase has been cloned. Transcription was not induced by heat shock so that trehalose accumulation was due probably to a post-translational activation of the enzyme (Fillinger *et al.*, 2001). Heat shock also induced the accumulation of mannitol and mRNA from the catalase gene CATA (Noventa-Jordao *et al.*, 1999). A SakA MAP kinase, equivalent to Hog1 in *S. cerevisiae*, has been described and implicated in sexual development and spore viability (Kawasaki *et al.*, 2002). A deletion mutant, SIK1, demonstrated a 60% reduction in hyphal extension rates in response to salt (Han & Prade, 2002).

In *A. niger* and *A. awamori* the genes coding the BiP protein homologues of *S. cerevisiae* have been cloned. Sequences resembling HSE and unfolded protein response elements, as found in the yeast KAR2 promoter, were present in the nontranscribed region of both genes (Hijarubia *et al.*, 1997; van Gemeren *et al.*, 1997). These genes were implicated in protein production and were induced by heat shock and by unfolded proteins (Punt *et al.*, 1998).

In *Aspergillus fumigatus*, early work dissected the antibody response in allergic bronchopulmonary aspergillosis, patients with an aspergilloma and patients with invasive aspergillosis by immunoblotting. This identified multiple antigens of molecular weight varying from 18 to 92 kDa. One antigen at 88 kDa was identified as an hsp90 homologue based on its cross-reaction with monoclonal antibodies against *C. albicans* and *A. ambisexualis* hsp90. Antibody against this antigen correlated with survival from invasive aspergillosis. The monoclonals also demonstrated the overproduction of a 50–52-kDa fragment in an aspergilloma (Burnie & Matthews, 1991; Matthews & Burnie, 1995; Matthews *et al.*, 1985). An immunodominant antigen of 88 kDa has been detected by sera from patients with an

aspergilloma (Kobayashi *et al.*, 1993; Tomee *et al.*, 1995), while rabbits seroconverted during infection to antigens of 41, 54 and 71 kDa (de Repentigny *et al.*, 1991).

In allergic bronchopulmonary aspergillosis, numerous antigens have been reported (Burnie, 1995). One of those has been identified as being an hsp90 homologue by screening a cDNA library of *A. fumigatus* with a pool of sera from patients (Kumar *et al.*, 1993). Expression of this sequence in *E. coli* produced the recombinant protein hsp1. This induced the expression of interleukin-2 and interferon- $\gamma$  and downregulated the production of IgE from the peripheral blood mononuclear cells cultured from patients with allergic bronchopulmonary aspergillosis (Murali *et al.*, 1998). This was delineated further to the level of individual epitopes (Svirshchevskaya *et al.*, 1998). An hsp70 homologue from *Penicillium citrinum* has also been identified as an allergen by screening a lambda Uni-Zap XR cDNA library with serum from an asthmatic patient (Shen *et al.*, 1997).

Genomic evidence has also been presented for mating abilities in *A. fumigatus* and a MAP kinase, Fus 3p, has been described (Poggeler, 2002). Paradoxically, transposon mutagenesis failed to identify any hsp as an essential gene (Firon *et al.*, 2003).

### ***Histoplasma capsulatum***

*Histoplasma capsulatum* grows in a mycelial form in soil or at 25 °C and as a parasite it grows as a yeast at 37 °C and in infected tissues. The pattern of hsp production was strain- and temperature-dependent, with maximum expression between 34 and 37 °C (Shearer *et al.*, 1987). This was confirmed for hsp70 and hsp82 independently and the individual genes were cloned (Minchiotti *et al.*, 1991, 1992; Caruso *et al.*, 1987). The ability of a strain to respond to a stress response was determined by the saturated to unsaturated fatty acid ratio of the cell membrane (Carratu *et al.*, 1996; Maresca & Kobayashi, 1993). The enzyme delta-9-desaturase, responsible for oleic acid production in the membrane, was inactive in the mycelia from *H. capsulatum* G217B at 25 °C, whereas it was active at the same temperature in the Down strain. The former isolate was thermo-tolerant while the latter was temperature-susceptible.

Monoclonal antibodies against hsp70 reacted against four bands at 32, 54, 66 and 80 kDa on immunoblot of an extract of *H. capsulatum* yeast cells. The 80-kDa protein was identified by direct amino acid sequencing as homologous to hsp70. Immunization of mice with this antigen produced host resistance to a sublethal but not lethal dose of yeasts (Gomez *et al.*, 1992; Gargano *et al.*, 1995).

A monoclonal antibody localized this antigen to the cell wall (Jeavons *et al.*, 1994). When expressed as a recombinant the molecule cross-reacted with a monoclonal antibody against human hsp70. Vaccination of mice with this

molecule, followed by intranasal challenge, failed to demonstrate protection, but it did induce a cell-mediated immune response (Allendoerfer *et al.*, 1996). A homologue of hsp60 has also been identified. Originally, this was isolated by electroelution and termed HIS-62. It was shown to induce protection against a lethal challenge with intravenous *H. capsulatum* yeast cells and was detected in proliferation assays by T cell clones from exposed humans (Gomez *et al.*, 1995). Vaccination of mice with recombinant clones expressing regions of the hsp60 protein delineated a conserved area between amino acids 172 and 443, against which a protective immune response was detected in both BALB/C and C57BL/6 mice (Deepe *et al.*, 1996). T cell clones from C57BL/6 mice vaccinated with this protective fragment of hsp60 were nearly 90% V $\beta$ 6<sup>+</sup> and the remainder were V $\beta$ 14<sup>+</sup>. Depletion of the V $\beta$ 6<sup>+</sup> cells and not the V $\beta$ 14<sup>+</sup> cells abrogated protection (Deepe & Gibbons, 2001). Immunization with the full protein produced a very different profile with expansion of the V $\beta$ 8.1/2<sup>+</sup> cells. There were common CDR3 sequences between the T cell clones isolated with a dominance of sequences containing a GG region (Scheckelhoff & Deepe, 2002).

A further examination of the response to immunization with the hsp60 fragment demonstrated that the V $\beta$ 6<sup>+</sup> cells had a Th1 profile, while there was also a small subpopulation of the V $\beta$ 8.1/2<sup>+</sup> T cells which had a Th2 profile. All Th2 cells accelerated mortality in a murine model while the ameliorative effect of the Th1 cells was neutralized by antibody to interferon- $\gamma$  (Deepe & Gibbons, 2002a). Following immunization of mice with hsp60, cytokine release was measured from spleen cells. There was substantially more interferon- $\gamma$ , interleukin-10 and interleukin-12 than found in mice immunized with recombinant hsp70 or bovine serum albumin. Treatment of the hsp60-immunized mice with a monoclonal antibody against either interferon- $\gamma$  or interleukin-12 abolished the ability of hsp60 vaccination to protect the mice (Deepe & Gibbons, 2002b). Hsp60 has been shown to be the surface molecule which binds to complement receptor type 3 on macrophages (Long *et al.*, 2003).

### ***Paracoccidioides brasiliensis***

The fungus grows as a mycelium at 26 °C and as unicellular yeast at 36 °C, which is important in the pathogenicity of the fungus. Initially an hsp70 homologue was identified as being induced by heat shock at 42 °C and by being expressed in the transition from the mycelial to the yeast phase. Northern blots confirmed its expression at a low level in the mycelial form. There was a high identity (89.2%) with the homologue identified from *H. capsulatum* and both sequences contained introns at the 5' and 3' ends. Immunoblotting with sera from infected patients identified a band at 87 kDa. This reacted with a monoclonal antibody against *H. capsu-*

*latum* hsp80. Direct amino acid sequencing demonstrated that, despite its aberrant molecule weight, it was an hsp70 homologue. The yeast to mycelium transition was associated with a decrease in expression, but immunochemical staining demonstrated *in vivo* expression (Diez *et al.*, 2002).

An hsp60 homologue has also been cloned and characterized. A single copy was shown by Southern blot analysis with three exons divided by two introns. Expression of hsp60 in *E. coli* produced a recombinant protein which reacted with sera from infected humans (Izacc *et al.*, 2001). Antibody against this was suggested subsequently as a potential surrogate marker of disease (Cunha *et al.*, 2002).

An hsp100 homologue, termed ClpB, was identified by screening a  $\lambda$  Dash II genomic library with a PCR fragment of 660 bp representing the chitinase gene of *Coccidioides immitis*. It was 46% identical to the *S. cerevisiae* homologue and was detected in the yeast phase (Jesuino *et al.*, 2002).

The application of expressed sequence tag analysis identified the expression of hsp82 and hsp104, ubiquitin and delta-9-desaturase at high levels in the yeast phase. In the transition from mycelium to yeast the genes representing the three stress proteins showed a fivefold increase at 5 h, followed by constant levels of expression and a decreased level after 48 h. Levels of delta-9-desaturase increased at the onset of the mycelium to yeast phase with a more constant expression in the yeast to mycelium transition (Goldman *et al.*, 2003).

### ***Trichophyton rubrum*, *Phycomyces blakesleeanus*, *Fusarium oxysporum*, *C. immitis* and *Pneumocystis jirovecii* (*P. carinii*)**

An hsp70 homologue has been cloned from a cDNA library derived from *T. rubrum*. The gene was expressed constitutively by cells cultured at 27 °C and upregulated strongly after culture at 37 °C (Rezaie *et al.*, 2000). In the zygomycetes, *P. blakesleeanus*, blue light-stimulated macrophorogenesis and induced a gene coding for an hsp100 homologue (Costantino *et al.*, 1994). In *F. oxysporum* seven hsps of 18, 35, 70, 74, 80, 83 and 95 kDa and a stress response gene STI35 (where disruption led to increased survival at 45 °C in minimal medium) have been identified (Thanonkeo *et al.*, 2000).

An hsp60 homologue has been cloned from *C. immitis*; the product exhibited a similarity of 78–83% to other fungal hsp60 proteins. Immunization of BALB/C mice led to the proliferation of T cells (Thomas *et al.*, 1997). Recombinant hsp60 was much less effective as a protective antigen than recombinant urease in murine models of infection. This was related to the ability of the recombinant urease to stimulate a Th1 response (Li *et al.*, 2001).

Hsp70 homologues have been cloned from *P. carinii* and *P. carinii rattus* with 83% DNA and 90% amino acid sequence identity. The genes were induced by heat shock,

glucose starvation, inhibition of protein transport and N-linked glycosylation (Stedman & Buck, 1996). Further, the gene PCSA1 was cloned from *P. carinii*, which belonged to the SSA family from *S. cerevisiae*, and from *P. jiroveci* a third set of homologues from the SSB family have been identified (Stedman *et al.*, 1998). A reverse transcriptase for detecting *P. jiroveci* in bronchoalveolar lavage fluids from patients with pneumonia, based on hsp70 sequences, has been described (Latouch *et al.*, 2001). The existence of a pheromone receptor *ste3* (Smulian *et al.*, 2001) and MAP kinase (Thomas *et al.*, 2001) suggest that hsp90 is important in *P. carinii*. Cloning of the *P. carinii* MAP kinase homologue PCM into *S. cerevisiae* restored the pheromone signal in *S. cerevisiae fus3Δ kss1Δ* mutants. The activity of PCM was significantly elevated in trophic forms compared with cysts (Vohra *et al.*, 2003).

## The postgenomic era

The availability of full or partial genome sequences for at least 16 fungi has opened a new approach (Giaever *et al.*, 2002). Instead of individual genes or proteins being studied, the simultaneous effects of a single event can now be studied. Changes in fungal morphology or stress induction by heat shock, toxic agents or antifungal drugs can be correlated with changes in the expression of multiple genes and proteins. Searching the databases with *S. cerevisiae* homologues can be used to delineate new hsps without having to characterize them individually. Their subsequent expression, combined with the production of mutants, can be used to delineate function. From a proteomics perspective, mass spectrometry has evolved as a versatile tool for examining the simultaneous expression of more than 1000 proteins and the identification of post-translational modifications. For both DNA and protein, the array format has allowed this to be performed on a single large scale (Zhu *et al.*, 2003). This section will focus on how these methodologies have elucidated the roles of hsps in *S. cerevisiae*, *C. albicans* and in the other pathogenic fungi.

## *Saccharomyces cerevisiae*

Microarray analysis of yeast open reading frames demonstrated upregulation by heat shock from 25 to 39 °C for 1 h of hsp12, hsp26, hsp30, SSA4, hsp60, SSA3, hsp78 and hsp82, whereas cold shock up-regulated YHR064, TIP1 and TIR1 (Lashkari *et al.*, 1997). The application of differential display of PCR-amplified reverse-transcribed mRNA (DDRT-PCR) (Sturtevant, 2000) demonstrated the up-regulation of hsp90 when cells were heat-shocked from 25 to 37 °C for 30 min (Gross & Watson, 1998).

Comparison of hsp expression in oxygen-limited conditions in the presence of either glucose or maltose demon-

strated that with growth in maltose hsp12, hsp26 and hsp30 were highly expressed (Donalies & Stahl, 2001). The effect of amphotericin B and nystatin on the expression of 5935 yeast genes demonstrated that hsp12 and hsp30 were upregulated (Zhang *et al.*, 2002). Amphotericin B was shown subsequently to upregulate hsp12, hsp26, SSA4 and hsp82. Caspofungin stimulated MAP kinase and hsp12, and fluorocytosine stimulated hsp12 and hsp26 (Agarwal *et al.*, 2003).

The application of proteomics with antibodies against the Ssa family recognized Ssa1, Ssa2, Ssb1 and Ssb2 on immunoblot after two-dimensional electrophoresis (Pardo *et al.*, 1999). Purification of 26S proteasome demonstrated its interaction with hsp70 and hsp82 (Verma *et al.*, 2000). The parallel and comparative analysis of the proteome and transcriptome of sorbic acid-stressed cells demonstrated the upregulation of hsp26, hsp42, SSA1 or SSA2, SSB1 or SSB2, SSC1 and SSC4. A deletion mutant of hsp26 was hypersensitive to sorbic acid (de Nobel *et al.*, 2001). To facilitate global protein analysis, a *S. cerevisiae* fusion library was created where each ORF was tagged with a high-affinity epitope and expressed (Ghaemmaghami *et al.*, 2003).

## *Candida albicans*

The application of antisense-based functional genomics to genes critical for the growth of *C. albicans* has identified 86 genes. None of these coded for an hsp (De Backer *et al.*, 2001b). DNA microarray studies did not implicate an hsp in filamentation (Lane *et al.*, 2001), while itraconazole down-regulated hsp104 and the genes coding for trehalose biosynthesis (De Backer *et al.*, 2001a; De Backer & Van Dijck, 2003). The application of DDRT-PCR identified seven genes induced in *C. albicans* during macrophage phagocytosis. This did not include an hsp (Prigneau *et al.*, 2003). The application of genomic arrays and a cDNA subtraction protocol to gene expression in cells exposed to human blood demonstrated downregulation initially of SSA4 and hsp6. Later there was upregulation of hsp6 and SSC1. SSA4 was upregulated more in *C. albicans* grown in whole blood than in plasma. SSB1 was upregulated early on and then down-regulated (Fradin *et al.*, 2003). The application of GRACE™ (gene replacement and conditional expression) identified 567 essential genes in *C. albicans* but did not identify a single hsp as essential (Roemer *et al.*, 2003).

Two-dimensional electrophoresis demonstrated that 25 proteins were upregulated in an azole-resistant strain, including the CYP51 gene coding for 14- $\alpha$ -lanosterol demethylase (Marichal *et al.*, 1997). The combination of three different extracts (cytoplasm, protoplast and secreted proteins) and immunoblotting with six sera identified at least 18 separate spots, including enolase, glyceraldehyde-3-phosphate dehydrogenase and a 34-kDa protein (Pitarch *et al.*, 1999). Sequential fractionation and two-dimensional



electrophoresis demonstrated that hsp90, ssa1, ssa4, ssb1, ssd1/kar2, szs1/pdr13 and hsp104 were cell surface-associated proteins. Hsp90 and ssa4 were induced by hyphal formation (Pitarch *et al.*, 2002). Hsp90 was identified subsequently in the microsomal fraction of *C. albicans* and expression was increased approximately threefold after a 2-h incubation with  $1 \mu\text{g mL}^{-1}$  of mulundocandin (Bruneau *et al.*, 2003). Examination of highly expressed proteins in the hyphae of *C. albicans* by two-dimensional electrophoresis failed to identify any hsps (Choi *et al.*, 2003). A recent proteomic map identified Ssc1, Ssb1 and hsp60 (Hernandez *et al.*, 2004). Strategies for dissecting the *C. albicans* proteome into different fractions have been proposed (Pitarch *et al.*, 2003b) and the COMPLUYEAST-2DPAGE database has identified Ssa1 and Ssb1 (Pitarch *et al.*, 2003a).

### Other pathogenic fungi

The SAGE has been applied to *C. neoformans* to determine which genes had higher transcript levels at 37 °C compared with 25 °C. This demonstrated upregulation of hsp60, hsp70 and hsp80 at 37 °C, whereas hsp12 was upregulated at 25 °C (Steen *et al.*, 2002).

DDRT-PCR has been applied to *H. capsulatum* to determine which genes were differentially expressed during macrophage infection. This did not identify any hsps (Colonna-Romano *et al.*, 1998).

### Therapeutic applications of hsps

The study of hsps in fungi cannot be performed in isolation as there is substantial homology with their counterparts in bacteria and mammals. In bacteria, gp96 peptide vaccination protected mice against *Listeria monocytogenes* infection (Zugel *et al.*, 2001). There was a humoral response against mycobacterial hsp65 in patients with constrictive pericarditis (Ng *et al.*, 1994). Hsp65 and hsp70 DNA vaccines followed by Bacilli Calmette–Guérin (BCG), induced protection in cattle against bovine tuberculosis (Skinner *et al.*, 2003). In *Helicobacter pylori*, hsp60 has been considered as a potential candidate for a subunit vaccine (Sharma *et al.*, 1997) and a long-lasting antibody response to hsp60 was demonstrated in animals infected by *Brucella abortus* (Galdiero *et al.*, 2000).

This is consistent with the dominance of hsp60 in infections because of *H. capsulatum* and *C. immitis* (Gomez *et al.*, 1992; Maresca & Kobayashi, 1993; Marsh *et al.*, 1998). The role of these molecules as potential vaccines is difficult to assess, as this research is at a preclinical stage.

In man, hsps including hsp60, hsp70, hsp90 and gp96, have been described as activators of the human innate immune system (Wallin *et al.*, 2002). They carry peptides derived from tumour cells and virus-infected cells to anti-

gen-presenting cells (APCs). Peptide uptake was mediated by receptors such as CD91 on the APC surface (Basu *et al.*, 2001). This was consistent with the model proposed by Matzinger where APCs were activated by ‘danger/alarm’ signals from injured cells, which included endogenous proteins such as hsps (Matzinger, 2002). The release of hsps in mammalian cells has been linked to necrotic but not apoptotic cell death (Basu *et al.*, 2000). Vaccination with hsp protein/peptide complexes has been tried as a means of treating renal cell carcinomas and malignant melanomas (Hoos & Levey, 2003).

In mycology, hsp90 has been identified as a potential target for immunotherapy. Hsp90 homologues have been identified in bacteria (htpG), in parasites, in protozoa and in man. The degree of conservation between these molecules means that the importance of hsp90 is not confined to fungal infection, as hsp90 is one of the danger signals discussed above. An antibody-based drug against a conserved epitope will cross-react with the corresponding molecule in the other species. This review will thus comment on the involvement of htpG in bacterial and hsp90 in parasitic and protozoan infection. It will discuss how the cross-reaction with human hsp90 might impact on treating systemic fungal disease in man.

### *Escherichia coli* HtpG

In *E. coli*, the predicted sequence of *htpG* was 42% identical to human hsp83 (Bardwell & Craig, 1987) and a deletion mutant was similar to wild type in its ability to survive starvation (Spence *et al.*, 1990). HtpG overexpression occurred in cells shocked at 45 °C when *E. coli* were grown in a complex medium but not in a simple glucose/mineral medium (Mason *et al.*, 1999). HtpG participates in the *de novo* protein folding of mildly stressed *E. coli* cells by expanding the ability of the DnaK–DnaJ–GrpE complex to interact with newly synthesized polypeptides (Thomas & Baneyx, 2000). In *Bacillus subtilis*, *htpG* induction was influenced by actual temperature, and not by temperature increment or the appearance of non-native proteins within the cytoplasm (Versteeg *et al.*, 2003).

In *Porphyromonas gingivalis*, antibody levels to an hsp90 homologue correlated with oral colonization and poor periodontal health. Heat stress induced a fivefold increase in expression in the 68-kDa parent molecule, while the 44-kDa band was constitutively expressed (Lopatin *et al.*, 1999). The 68-kDa polypeptide was localized to the cytosolic fraction and the 44-kDa protein was associated with the membrane and vesicle fractions (Lopatin *et al.*, 2000). Raised levels of antibody to hsp70 and hsp90 were also demonstrated in diabetic patients with periodontitis (Sims *et al.*, 2002). The disruption of the *htpG* gene did not significantly affect growth and produced no effect on

adherence to cultured human cells (Sweier *et al.*, 2003). In *Actinobacillus actinomycetemcomitans* at 42 °C, an *hspG* insertion mutant grew at about half the rate of the parent strain (Sweier *et al.*, 2003). In *Corynebacterium jeikeium* septicaemias recovery from infection was associated with antibody on immunoblot against bands at 50, 51 and 110 kDa (Clark *et al.*, 1990). The 52-kDa band cross-reacted with a monoclonal and polyclonal serum raised against candidal hsp90 and a reverse passive latex agglutination test for detecting *Candida* antigen in disseminated candidiasis gave false positives in patients with *C. jeikeium* infection (Johnson *et al.*, 1989).

### Hsp90 and parasites and protozoa

Surface-expressed hsp90 has been shown to be immunogenic in Chagas' disease, ascariasis, leishmaniasis, toxoplasmosis, *Trichinella spiralis* and infection because of *Schistosoma mansoni* (Dragon *et al.*, 1985; Matthews, 1991; Kumari *et al.*, 1994; Streit *et al.*, 1996; Rojas *et al.*, 2000; Martinez *et al.*, 2001). *Trypanosoma cruzi* hsp90 can functionally complement yeasts (Palmer *et al.*, 1995), and its inhibition by the hsp90 inhibitor geldanamycin demonstrated that it was essential for cell division, as epimastigotes were arrested in the G1 phase of cell cycle (Graefe *et al.*, 2002).

Recombinant *Leishmania* hsp90 and hsp70 were recognized by sera from patients with visceral leishmaniasis but not by Chagas' disease patients (de Andrade *et al.*, 1992). Hsp90 inhibitors led to growth arrest and the differentiation from the promastigote to amastigote (Wiesiggl & Clos, 2001). Hsp70 and hsp83 have been suggested as distracting the immune system to a nonspecific activation of immune cells leading to immunosuppression. This was consistent with the observation that recombinant hsp70 and hsp83 fused to *E. coli* maltose-binding protein behaved as T cell-independent B cell mitogens in mice (Rico *et al.*, 2002). Two-dimensional electrophoresis demonstrated a similar change in protein profile following heat shock, acid pH and hsp90 inhibition (Bente *et al.*, 2003). In malaria, the HSP90 genes were translated predominantly in the ring and trophozoite stages. This persisted through much of the schizont stage and disappeared only in late schizonts. Geldanamycin was shown to have antimalarial activity (Banumathy *et al.*, 2003; Kumar *et al.*, 2003). Antibodies specific to hsp90 were remarkably increased in patients with malaria and in murine models (Kumar *et al.*, 1990; Zhang *et al.*, 2001). Antibodies against a *Plasmodium falciparum* protein fraction containing hsp90-protected squirrel monkeys against infection with parasites in the asexual stage (Bonney *et al.*, 1994).

### Hsp90 and antibodies in fungal infection

An immunodominant antigen of apparent molecular mass varying from 45 to 52 kDa was repeatedly demonstrated in

patients with serious infection because of *C. albicans* (Matthews *et al.*, 1984, 1987, 1988a; Strockbine *et al.*, 1984; Au-Young *et al.*, 1985; Neale *et al.*, 1987; Ferreira *et al.*, 1990; Porsius *et al.*, 1990; Peterson *et al.*, 1996; Weis *et al.*, 1997). Identification of this antigen has revealed two different proteins of apparent molecular mass 47 and 4 kDa. The protein of 48 kDa was characterized as enolase (Mason *et al.*, 1989) and the identity was confirmed by two-dimensional electrophoresis, although this approach demonstrated numerous proteins with similar electrophoretic properties (Pitarch *et al.*, 2002). Enolase was exploited as the basis of a diagnostic test (Walsh *et al.*, 1991). It was demonstrated as binding to human plasminogen (Jong *et al.*, 2003) and downregulated in fluconazole-resistant strains (Angiolella *et al.*, 2002). It was immunodominant in murine infection (Pitarch *et al.*, 2001), although antibody against enolase was only partially protective in a mouse model of infection (van Deventer *et al.*, 1996). It has been described as a source of autoantibody (Gitlits *et al.*, 2001) and a potential fungal allergen (Savolainen *et al.*, 1998).

The second, an antigen of apparent mass 47 kDa, was identified as the carboxy fragment of *C. albicans* hsp90 (Cho *et al.*, 2003). It was more abundant and could be distinguished from enolase by the pattern of cross-reactivity with an enolase-specific monoclonal antibody (Franklyn & Warming, 1994). Debate on the molecular size of the parent molecule has been centred on a predicted molecule mass of 81 kDa (Bromuro *et al.*, 1998; Sandini *et al.*, 2002). When it was cloned into *S. cerevisiae* it ran on gel electrophoresis at approximately 90 kDa. A mouse monoclonal antibody against it reacted with a band at 92 kDa (Matthews, 1992; Eroles *et al.*, 1997) and the apparent molecular mass on two-dimensional electrophoresis was higher than 81 kDa (Pitarch *et al.*, 2002). Hsp90, both in *S. cerevisiae* and *C. albicans*, was shown to generate fragments of 47 and 72–76 kDa on electrophoresis. This partial degradation was suggested as occurring in viable cells and accentuated when yeasts were transferred from fermentative to respiratory media (Eroles *et al.*, 1997). Heat-inducible ATP-binding proteins of 70–72 and 74–76 kDa from *C. albicans* have been recognized by the sera of infected patients. This is consistent with the 72–76-kDa protein retaining the amino-end ATP-binding site, which bound geldanamycin (Grenert *et al.*, 1997), while this was lost in the 47-kDa fragment (Swoboda *et al.*, 1993).

Hsp90 has been localized by staining with a specific polyclonal antibody to the cell wall surface while the 47- and 72-kDa proteins were also present in the cytoplasm (Burt *et al.*, 2003). An affinity-purified antibody against the 47-kDa antigen demonstrated that it was present in the cytoplasm and the cell wall of both yeast and mycelial cells (Matthews *et al.*, 1988b).

Hsp90 has also been detected on the cell surfaces of the hyphae (Urban *et al.*, 2003). It was hypothesized that the

47-kDa fragment was generated by *ycaB*, the *C. albicans* variant of the *S. cerevisiae* *cerevisin* (protease B). This would hydrolyse hsp90 at amino acid positions 59 and 78 to produce the 72-kDa fragment and at position 313 to produce the 47-kDa fragment (Burt *et al.*, 2003). A similar picture has been suggested for HtpG, in which hydrolysis at amino acid position 336 produced fragments from the 70-kDa parent molecule of 32 and 39 kDa (Nemoto *et al.*, 2001a). In *Leishmania donovani*, the theoretical molecular mass of hsp90 was 80 kDa, but the experimental mass was 85 kDa and other bands were described at 43 and 50 kDa (Bente *et al.*, 2003).

The 47-kDa antigen was immunodominant in patients with chronic mucocutaneous candidiasis and AIDS (Burford-Mason *et al.*, 1987; Matthews *et al.*, 1988a). In patients with surgical sepsis a statistically significant ( $P < 0.05$ ) correlate was reported with deep, rather than superficial, infection and it has been found in the sera of intensive-care patients with abdominal sepsis (Neale *et al.*, 1987; Weis *et al.*, 1997). Antibody to the 47-kDa antigen correlated with survival, whereas lack – or falling levels – of antigen were associated with a poor prognosis (Matthews *et al.*, 1984, 1987). A similar immunodominant molecule has been demonstrated in *Candida parapsilosis*, *C. glabrata*, *C. tropicalis*, *Candida krusei* and *A. fumigatus* (Matthews *et al.*, 1988a, 1990; de Belder *et al.*, 1989; Burnie & Matthews, 1991; Matthews & Burnie, 1995; Zhang *et al.*, 2000).

### Epitope mapping

The antibody response to hsp90 has been dissected to the level of individual epitopes by epitope mapping. This is the process where a set of polyethylene pins are created with overlapping nonapeptides such that peptide 1 consisted of residues 1–9, peptide 2 of residues 2–10, etc. Sera are tested in an enzyme immunoassay against these pins, which allowed the delineation of individual linear epitopes.

Sera from patients who had recovered from disseminated candidiasis and were antibody positive to the 47-kDa protein were tested against pins which represented this sequence. This identified two dominant epitopes near the amino end of the fragment, represented by LSREM and LKVIRK (Matthews *et al.*, 1991b). These were near the postulated cleavage site of *Candida* hsp90 and *E. coli* htpG (Nemoto *et al.*, 2001a; Burt *et al.*, 2003). These sequences were either identical or highly conserved, with the corresponding area in other pathogenic fungi such as *C. tropicalis*, *C. krusei* and *C. neoformans* and man. The third most immunodominant epitope was the species-specific DEPAGE just before the carboxy end (Burnie & Matthews, 2003).

Examination of sera from patients with disseminated candidiasis, invasive aspergillosis or systemic lupus erythematosus against the sequence representing human hsp90-

defined 12 epitopes, of which four were conserved with human hsp90. LKVIRK produced a humoral response in patients who recovered from disseminated candidiasis, invasive aspergillosis and malaria, but not in patients who died from an invasive mycosis or who had systemic lupus erythematosus. KIRY and>NNLGTI were antigenic in disseminated candidiasis and WSAN was immunodominant in invasive aspergillosis (al-Dughaym *et al.*, 1994). KIRY has been implicated as part of the amino-end ATP-binding domain in malarial hsp90 (Banumathy *et al.*, 2003).

The amino acid sequences of hsp90 and HtpG, as discussed previously, have been subdivided into Regions A, B and C (Fig. 1) (Nemoto *et al.*, 2001a; Matsumoto *et al.*, 2002). A mutant 5CGZ HIS was created in *S. cerevisiae* which grew at 37 °C on galactose but not on glucose-containing plates. This defect was corrected by inserting constitutively expressed hsp90, but persisted when either the end of Region A overlapping LSREM or the beginning of Region B overlapping KILKVIRK was deleted (Burnie & Matthews, 2004). In htpG, Region B interacted with Region A from the same polypeptide and with Region C from a second molecule of htpG during dimerization (Nemoto *et al.*, 2001a). Sequences from htpG could replace Region B from hsp90  $\alpha$  and maintain the Region A relationship. Replacement impaired the interaction with Region C, again emphasizing the species-specificity of that area. The complex between Region A and Region B was labile at high temperatures, whereas the binding to Region C was stable even at 70 °C (Matthews *et al.*, 1991b; Tanaka *et al.*, 2001). In htpG, the self-oligomerizing and substrate-binding activities were both located in a single area in Region A (Nemoto *et al.*, 2001b). A second client-binding area responsible for dimeric configuration was demonstrated in the hydrophobic segment of the carboxy-terminal domain (Yamada *et al.*, 2003). This may, in *C. albicans*, involve the species-specific epitope DEPAGE.

In *S. cerevisiae*, the unmasking of the second cryptic ATP-binding site at the carboxy end was dependent on the sequence QQSKILKVI at the start of Region B (Soti *et al.*, 2002). This again underlined the importance of the amino end of Region B to the function of the molecule. These studies all support the potential for the sequence LSREMQQNKILKVIRK as a binding area for an antibody fragment which would inhibit the function of the parent molecule and cross-react with the 47-kDa fragment (Matthews *et al.*, 2003; Burnie & Matthews, 2004). The conservation with the corresponding region in human hsp90 raised the question of whether hsp90 released from necrotic human tissues would act in concert with fungal hsp90 to produce disease. The potential effect of free circulating hsp90 on human physiological processes needs to be considered.

## Hsp90 and nitric oxide

In 1998, hsp90 was demonstrated as interacting with endothelial nitric oxide synthase (eNOS) leading to the release of nitric oxide (NO) (Garcia-Cardena *et al.*, 1998). Nitric oxide has been shown to be a smooth muscle vasodilator and a potent inhibitor of platelet aggregation (Ignarro, 2002). Overexpression of hsp90 increased NOS activity and led to phosphorylation and NO release. Inhibition of the ATPase function by geldanamycin blocked acetylcholine-induced endothelium-derived NO production in human volunteers (Shastry, 2002). The excessive NO production observed in portal hypertension in animals has been shown to be mediated in part through hsp90 signalling (Shah *et al.*, 1999). The vasodilator effect of NO agonists has been confirmed in a rabbit model for determining microvascular anastomotic patency rates (Chiang *et al.*, 2003).

Oestradiol caused endothelium-dependent vasodilation mediated, in part, by NO. Hsp90 played an important role in this oestrogen-receptor-modulated activation of eNOS (Russell *et al.*, 2000). The activation of eNOS was orchestrated by an interaction between hsp90 and the protein kinase Akt, which led to phosphorylation of the serine at amino acid position 1179 in eNOS. There was preferential binding of the B region of hsp90 overlapping the NKILK-VIRKNIVKK epitope to eNOS when compared with constructs representing the A and C regions (Fontana *et al.*, 2002). Inducible NO synthase (iNOS) was absent from cells under normal conditions. Expression was induced by inflammatory mediators including various cytokines and microbial products (Nathan & Shiloh, 2000). Overexpression of hsp90 enhanced this process, while hsp90 inhibition dramatically decreased NO formation from iNOS in macrophages, which prevented cell damage (Yoshida & Xia, 2003).

Hsp90 activated the kinin-forming cascade leading to bradykinin release in the fluid phase and along cell surfaces. This involved the interaction of factor XII, prekallikrein and high MW kininogen in a zinc-dependent manner (Joseph *et al.*, 2002a). Zinc has been proposed previously as the bridge between hsp90 and the androgen receptor in the 8S complex. This involved a leucine-rich zipper region in hsp90 which overlapped the NKLK-VIRKNIVKK (Schwartz & Mizukami, 1991; Schwartz *et al.*, 1993). This activation was inhibited by a polyclonal antibody to hsp90.

Bradykinin has been described as a major mediator of swelling in C1 inhibitor deficiency and responsible for the angioedema seen with ACE inhibitors. It is a gastrointestinal vasodilator and released from mast cells during asthma (Joseph *et al.*, 2002a, b). The plasma kallikrein-kinin system has been proposed as a physiological counterbalance to the plasma rennin-angiotensin system, which would act by lowering blood pressure and preventing thrombosis. In a pathological situation, such as in disseminated candidiasis,

overexpression could produce a state mimicking septic shock (Shariat-Madar *et al.*, 2002). Increased levels of NO have been shown in a murine model of oral candidiasis (Elahi *et al.*, 2001) and in paracoccidioidomycosis NO was demonstrated as essential for resistance. Overproduction was associated with an increased susceptibility to the infection (Nascimento *et al.*, 2002).

## Mycograb<sup>®</sup>

The idea of using antibodies to treat disseminated candidiasis has existed since 1946, with a case report supporting it (Hiatt & Martin, 1946). The concept of combination therapy for infection involving immune response molecules and antimicrobial drugs has been advocated (Hengel & Masihi, 2003). This raised issues which could be subdivided into pharmacokinetic, relating to direct or indirect interactions affecting the drug concentrations at the site of infection, and pharmacodynamic, involving spectrum, synergism or antagonism, resistance and toxicity (Lewis & Kontoyianis, 2001).

The immunodominance of hsp90 in patients who survived disseminated candidiasis and invasive aspergillosis made this molecule a natural target for immunotherapy. Immunization with a membrane fraction from *C. albicans* containing hsp90 in combination with fluconazole protected mice from infection (Mizutani *et al.*, 2000). A virulence-associated immunomodulation protein secreted by *C. albicans* was responsible for immunoprotection against candidiasis after the spontaneous healing of mice inoculated with 10<sup>6</sup> blastoconidia (Tavares *et al.*, 2003). This molecule was a B cell mitogen, a feature of *L. donovani* hsp90, suggesting that it was related to the 47-kDa fragment of candidal hsp90 (Tavares *et al.*, 1993; Kumar *et al.*, 2003). Immunoenhancement potentiated the effect of amphotericin B on *L. donovani* (Murray *et al.*, 2003) and human sera improved the *in vitro* activity of caspofungin against *A. fumigatus* (Chiller *et al.*, 2000).

The perfect combination therapy would involve two compounds with intrinsic antifungal activity where synergy could be demonstrated *in vitro*, in animal models of infection and in human studies. There would be an improvement in clinical outcome and a reduction in the incidence of resistance. The problem with mixing conventional antifungal drugs is that there is likely to be an increase in toxicity either directly or indirectly by interfering with the other medications needed to support the patient. An antibody-based therapy has the ability to avoid this, as all patients have antibody and this represents a molecule which the patient would make naturally on recovery.

In the case of hsp90, initial studies in mice involved human sera from patients who were antibody-positive to the 47-kDa antigen and had recovered from the infection, rabbit

polyclonal serum, and a mouse monoclonal antibody against the epitope LKVIRK. The sera from the infected patients halved mortality and this effect was mediated by the immunoglobulin fraction and not observed with normal serum. The rabbit antiserum reduced the mortality to 83% and lacked antibody to the LKVIRK epitope, despite a high titre of antibody against hsp90. The mouse monoclonal antibody reduced mortality to 66% (Matthews *et al.*, 1991a). A human recombinant antibody against LKVIRK was assessed in acute and chronic models of murine invasive candidiasis. In the lethal model, intravenous challenge with both a fluconazole-sensitive and -resistant strain, followed 2 h later by a single dose of recombinant antibody, reduced mortality by  $\geq 40\%$ . In the chronic model infected with the fluconazole-sensitive strain, the recombinant antibody improved the rate of renal clearance (Matthews *et al.*, 1995).

The availability of rapid gene sequencing allowed the sequencing of all the antibody genes from the peripheral B cells of patients as they recovered from a specific infection (FABTEC<sup>®</sup>, www.neutecpharma.com). In disseminated candidiasis examination of the sequences demonstrates focusing, most obviously in the CDR3 region of the V<sub>H</sub> chain. Six V<sub>H</sub> chains accounted for 72% of the clones. These were resynthesized in *E. coli* as a polyhistidine-tagged genetically recombinant antibody. One of these bound to NKILKVIRKNIV and was the basis of Mycograb<sup>®</sup>. It was produced by batch fermentation and purified to > 99% purity by three step-chromatography (Burnie & Matthews, 2004).

In preclinical assessment, Mycograb<sup>®</sup> showed activity against a wide range of yeast species, with MICs varying from 128 to 256  $\mu\text{g mL}^{-1}$ . This included fluconazole-sensitive and -resistant *C. albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis*. Mycograb<sup>®</sup> at 4 or 8  $\mu\text{g mL}^{-1}$  showed synergy with amphotericin B, and produced a fractional inhibitory index from 0.09 to 0.31. In a subacute murine model, Mycograb<sup>®</sup> at 2  $\text{mg kg}^{-1}$  produced a statistically significant improvement in either mean colony counts (Scheffé's test,  $P < 0.05$ ) or the number of negative biopsies (Fisher's exact test,  $P < 0.05$ ). Synergy occurred with amphotericin B at 0.6  $\text{mg kg}^{-1}$ , with both drugs needed for complete resolution of infection because of *C. albicans*, *C. krusei* or *C. glabrata* (Matthews *et al.*, 2003). Synergy with amphotericin B-resistant strains of *C. albicans* and *C. lusitanae* has also been demonstrated (G. Rigg, R. Mathews and J. Burnie, unpublished data). Murine kinetics at 2  $\text{mg kg}^{-1}$  produced a maximum concentration of 4.7  $\mu\text{g mL}^{-1}$ , a half-life  $\alpha$  phase of 3.75 min and a half-life  $\beta$  phase of 2.34 h (Matthews *et al.*, 2003). Mycograb<sup>®</sup> was evaluated against eight clinical isolates of *C. neoformans*. There was synergy between Mycograb<sup>®</sup> and amphotericin B as shown by a fractional inhibitory index from 0.27 to 0.31, and time-kill experiments (Nooney *et al.*, 2005).

In a first clinical trial in patients with disseminated candidiasis on amphotericin B, escalating doses of Mycograb<sup>®</sup> (from a test dose of 0.1 to 1  $\text{mg kg}^{-1} \text{ day}^{-1}$  and then 1  $\text{mg kg}^{-1}$  twice daily) were given. No evidence of side effects was seen and there was anecdotal evidence that the combination was therapeutically useful. In a further 10 patients given Mycograb<sup>®</sup> at 1  $\text{mg kg}^{-1}$  twice daily for 5 days, the peak levels varied from 6.6 to 9.9  $\mu\text{g mL}^{-1}$  (Matthews & Burnie, 2004). One hundred and thirty-nine cases of invasive candidiasis were treated with liposomal amphotericin B in combination with a 5-day course of Mycograb<sup>®</sup> at 1  $\text{mg kg}^{-1}$  twice daily or placebo (saline). An 84% overall response rate, vs. 48% in the placebo control group, was reported. Analysis of the clinical response and mycological response (i.e. culture confirmed clearance of the infection in the laboratory at day 10) showed a highly statistically significant difference between the two groups ( $P$ -value < 0.001). There was a *Candida*-attributable mortality of 18% in the placebo group (receiving Amphotericin B and saline) compared with 4% in the group receiving Amphotericin B and Mycograb<sup>®</sup> ( $P$ -value < 0.025). In combination with amphotericin B, Mycograb<sup>®</sup> will be useful for the treatment of invasive candidiasis in immunocompromised intensive care patients.

## Conclusion

This review has demonstrated that the depth of knowledge of hsp is greatest for *S. cerevisiae*, while the study of hsp in the other fungi is still at an early stage. Several trends have emerged. A fungus may have a multitude of hsp of similar molecular mass, e.g. hsp70 in *S. cerevisiae*; these may have been thought initially to have the same function but further analysis has revealed their uniqueness (Frydman, 2001). This suggests that the identification of multiple hsp in the pathogenic fungi when the genomes have been analysed will be followed by the description of specific functions for each of them.

The ability to express hsp at a given temperature may be strain-specific and, as with *H. capsulatum*, this may be a virulence factor (Kumar *et al.*, 1993). This mitigates against the study of only a single isolate as a way of determining the heat-shock response of a species.

A sexual pathway has been described for many of the pathogenic fungi *C. albicans*, *C. glabrata* and *A. fumigatus* (de Repentigny *et al.*, 1991; Malloy *et al.*, 1993; Yazdanyar *et al.*, 2001). The description of pheromones for *C. albicans*, *S. cerevisiae* and *A. ambisexualis* (Brunt & Silver, 1986; Malloy *et al.*, 1993; Louvion *et al.*, 1998) suggests that these are widespread, and may form part of a quorum-type fusing mechanism, enabling the fungus to react to the outside environment. This may involve the transition to a form capable of causing invasive disease, for example the yeast

phase in *H. capsulatum* (Kumar et al., 1993) and *P. brasiliensis* (Deepe et al., 1996). This response may be controlled by the ratio of saturated to unsaturated fatty acid with the enzyme  $\Delta$ -a-desaturase implicated in *H. capsulatum* (Firon et al., 2003) and *P. brasiliensis* (Deepe & Gibbons, 2002b). Hsp90 involvement with the pheromone response has been demonstrated in *S. cerevisiae* (Louvion et al., 1998) and this is consistent with its presence on the cell surfaces of hyphae in *C. albicans* (Matthews, 1992).

Trehalose and HSFs have been recognized as important in *S. cerevisiae*, but these molecules have not been examined extensively in the pathogenic fungi. The impact of genomics and proteomics has been that they have allowed the effect of noxious stimuli on multiple fungal targets to be indicated simultaneously. This work will be extended to look at the effect of combinations of antifungal drugs, so that synergy, indifference and antagonism can be dissected down to the level of an individual gene or protein. The dominance of hsp as molecular chaperones suggests that changes in their expression will be an important part of this process.

Preclinical studies with Mycograb<sup>®</sup> demonstrated synergy with amphotericin B, which was reflected in patients with disseminated candidiasis, paving the way for a rational combination therapy in systemic fungal infection. The future should thus see fresh therapeutic opportunities combined with a rapid expansion in knowledge.

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