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A yeast-based assay reveals a functional defect of the Q488H polymorphism in human Hsp90α

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Abstract

It has been argued that the molecular chaperone Hsp90 guards the organism against genetic variations by stabilizing variant Hsp90 substrate proteins. However, little is known about polymorphisms affecting its own functions. We have followed up on a recent study describing two polymorphisms that alter the amino acid sequences of the two Hsp90 isoforms Hsp90α and Hsp90β. Hsp90 is essential for cell proliferation in the budding yeast *Saccharomyces cerevisiae*, but the human proteins can replace the endogenous ones. In this growth assay, the variant V656M of Hsp90β was indistinguishable from wild-type. In contrast, the Hsp90α variant Q488H, which carries an alteration of a very highly conserved residue, was severely defective for growth compared to wild-type Hsp90α. Hence, the characteristics of this yeast-based system—simplicity, rapidity, low cost—make it ideal for phenotype screening of polymorphisms in *HSP90* and possibly many other human genes.

Keywords: Hsp90; Molecular chaperone; Polymorphism; Human mutation; *Saccharomyces cerevisiae*; Yeast

The ubiquitous molecular chaperone Hsp90 is essential in all eukaryotes. It is abundant at normal physiological temperatures, accounting for approximately 1–2% of total cellular proteins, and further induced by stress to respond to problems in protein folding [1,2]. Hsp90 interacts with a large, diverse but selective set of substrate proteins, including many kinases and transcription factors, influencing their activity and stability. Therefore, Hsp90 along with other molecular chaperones may have a crucial role in responding to disease and ageing [3,4]. The observation that tumour cells are particularly vulnerable to pharmacological inhibition of Hsp90 has led to clinical trials with Hsp90 inhibitors as potential anti-cancer agents [5,6].

Polymorphisms are widespread throughout the genome and can have an impact on complex traits such as susceptibility or resistance to diseases. Some polymorphisms occurring in protein coding regions are not always evident under normal conditions because Hsp90 can buffer against the effects of genetic variation by stabilizing or assisting a variant protein. This buffering allows for the accumulation of cryptic polymorphisms until they become unleashed by a pathological or environmental challenge [4,7,8]. Likewise, a polymorphism of Hsp90 itself could have an impact on many processes such as human diseases and ageing.

Humans, like most species, have two Hsp90 isoforms, Hsp90α and Hsp90β, which are about 85% identical at the protein level. They are encoded by two separate genes, *HSPCA* and *HSPCB*, respectively. While the expression of the two genes is regulated differently, a clear functional difference of the two protein isoforms in human cells has yet to be identified.

As of today, there have only been two studies on polymorphisms in the *HSP90* genes [9,10]. In a survey of samples from 73 Caucasians [9], a total of 29 genetic variants were found, but only 3 could be expected to alter the gene product. One of these results in a frame-shift and encodes a severely truncated product that would be non-functional if stably expressed. The two others lead to amino acid changes, Q488H and V656M in Hsp90α and Hsp90β,

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respectively. In the more recent smaller study [10], the authors could not link Hsp90 polymorphism with varicocele-associated male infertility although they did find three silent changes. In light of the pivotal role of Hsp90 in many cellular processes, it is of great interest to assess the functionality of genetic variations, and notably those that alter the gene product. One way to approach this question is to reintroduce these variant Hsp90 proteins into a biological assay system. This is what we have undertaken here by testing whether these variants can support Hsp90-dependent growth in yeast.

Materials and methods

Plasmids and strain construction. The human *HSP90α* coding sequences were cloned into plasmid pRS313/GPD-PGK (a gift from A. Kralli), a low copy number CEN/ARS yeast expression vector with a *HIS3* marker derived from pRS313 [11], to generate pRS313/Hsp90α, *HSP90β* was expressed from pHCA/hHsp90β [12]. Point mutations G1464C and G1966A (numbering relative to AUG) were generated in *HSP90α* and *HSP90β*, respectively, by site-directed PCR mutagenesis to produce plasmids pRS313/Hsp90α Q488H and pHCA/hHsp90β V656M. The control plasmid pHCA/hsp82 [12] contains the yeast *HSP82* open reading frame.

All genes are under the control of the strong constitutive promoter from the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene. The yeast strain pp30#10 (MATa *trp1-289 leu2-3,112 his3-200 ura3-52 ade2-101^{oc} lys2-801^{am} Δhsc82::KanMX4 Δhsp82::KanMX4*) containing the *HSC82* gene in a 2μ episome with the *URA3* gene [13] was transformed by the lithium acetate/polyethylene glycol method [14].

Cell growth assays. Yeast cells were cultured in minimal SD media (0.67% yeast nitrogen base without amino acids) supplemented with 2% glucose and essential amino acids and nucleotides. Starter cultures were used to inoculate larger volumes and then cells were grown with shaking at 30 °C until mid-logarithmic phase was reached ($OD_{600} = 0.9$). Cells harvested by centrifugation and washed with water were either processed for protein extraction and analysis or for growth assays. For growth assays, cells were serially diluted by steps of 10 and 5 μl of each dilution was spotted onto solid media. These contained 20 g of Bacto-agar per litre and 2% glucose, and were formulated either as minimal SD medium or rich YPD medium (10 g yeast extract, 20 g Bacto-peptone per litre). Where indicated, 5-fluoro-orotic acid (FOA) was added to select for the loss of the episome containing *HSC82*.

Immunoblotting. Cell extracts were prepared as described previously [15]. After quantification with the Bio-Rad Bradford reagent, 40 μg was loaded onto 7.5% SDS–polyacrylamide gels. To confirm that equal amounts of protein had been loaded, proteins were stained with Ponceau S after transfer onto a nitrocellulose membrane before immunostaining. The blot was probed either with chicken antibodies raised against recombinant yeast Hsp82 (used at 1:500 dilution) [15] or with mouse monoclonal antibodies AC88 or H90-10 (gifts from David Toft) used at 1:1000 dilution to recognize human Hsp90α or Hsp90β, respectively.

Results and discussion

In this study, we explored the phenotype of two *HSP90* polymorphisms identified in humans [9] using the budding yeast *Saccharomyces cerevisiae*. Budding and fission yeasts are the only organisms amenable to investigate Hsp90 function in vivo relatively easily because their genomes can easily be manipulated. The essential endogenous *S. cerevisiae* genes encoding Hsp90, *HSC82*, and *HSP82*, can be removed, provided cell viability is maintained by an episomally expressed copy of either gene [16]. This plasmid can be exchanged by another plasmid driving expression of human Hsp90, which complements a double mutant yeast strain at least for cell growth [17–19].

The polymorphism Q488H in human Hsp90α is a non-conservative substitution of a neutral, polar amino acid to a basic residue. Fig. 1 shows a sequence alignment of the portion surrounding this polymorphism (top panel). This glutamine residue is perfectly conserved from *E. coli* to humans and in both yeast Hsp82 and Hsc82. This is in contrast to the polymorphism V656M in Hsp90β, which occurs in the C-terminal dimerization domain [20–23] in a residue that is poorly conserved in evolution and between isoforms within the same species (Fig. 1, lower panel).

Plasmids expressing either a wild-type isoform of human Hsp90 or a polymorphic variant were expressed in yeast.

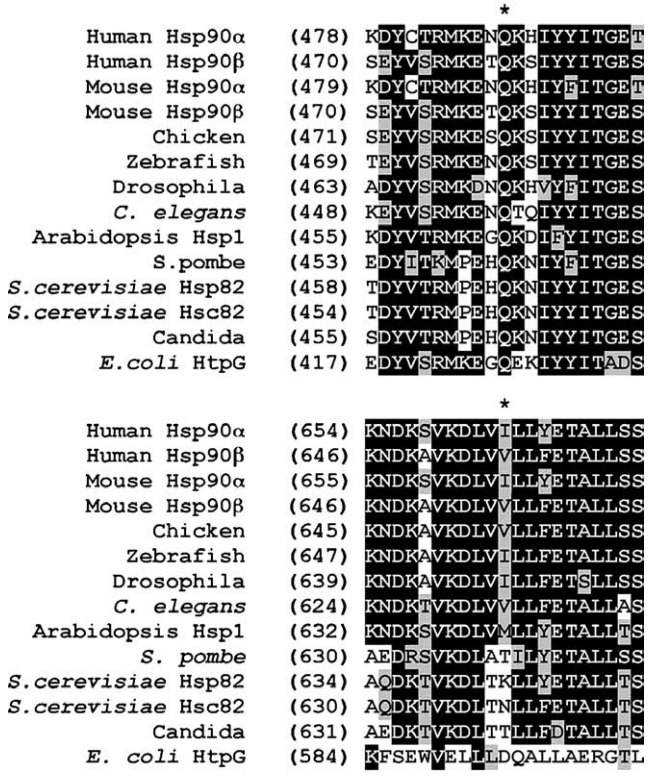

Fig. 1. Sequence alignment of the polymorphic portions of Hsp90. Amino acid sequences from several Hsp90 sequences were aligned using the sequence alignment program Clustal. The alignments around the Q488H and V656M polymorphic sequences are shown in the top and bottom panels, respectively. Identical residues are boxed in black, conserved substitutions are shaded in grey. The polymorphic residues investigated are indicated with an asterisk. The residue Q488 is conserved amongst all species, while the residue V656 is only semi-conserved. Aligned sequences: human Hsp90α (Accession Nod. NM_005348) and Hsp90β (NM_007355), mouse Hsp90α (NP_034610) and Hsp90β (NP_032328), chicken Hsp90β (NM_206959), zebrafish Hsp90β (AAH65359), *Drosophila* Hsp83 (P02828), *Caenorhabditis elegans* C47E8.5 (CAA99793), plant *Arabidopsis thaliana* Hsp1 (BAA98982), *Schizosaccharomyces pombe* Swo1 (CAB54152), *Saccharomyces cerevisiae* Hsp82 (A73596) and Hsc82 (M26044), *Candida albicans* Hsp90 (CAA56931), and *Escherichia coli* HtpG (BAB33949).

Yeast cells expressing both yeast *HSC82* to ensure viability and a human *HSP90* were grown to exponential phase in liquid media, and then serial dilutions were spotted onto solid media containing the drug FOA to select for loss of the plasmid expressing yeast *HSC82*. Expression of human Hsp90 in yeast produces no observable dominant negative effects on growth (Fig. 2, top left). As expected, yeast cells forced to eliminate all yeast Hsp90 and to rely on heterologous expression of human Hsp90 can survive (Fig. 2). However, it is noteworthy that cells expressing only Hsp90α grow slightly more slowly than wild-type cells (Fig. 2, top right), indicating that human Hsp90α cannot fully substitute for yeast Hsp90 even under standard growth conditions. With human Hsp90β, yeast growth appears to be similar to that of wild-type cells.

Introducing the non-conservative substitution Q488H into Hsp90α results in growth that is even worse than with wild-type Hsp90α, while the conservative substitution V656M Hsp90β has no adverse effect on yeast growth (Fig. 2). Following complete removal of all yeast Hsp90 genes by selection on FOA plates, cells expressing only human *HSP90* (wild-type or polymorphic) were isolated so that their growth properties could be examined under different conditions. Unlike the starting strains, these strains could be assayed without a concomitant requirement to select for loss of the yeast *HSC82* gene. This time, cells were spotted onto rich and minimal media, and grown at either 30 or 37 °C (Fig. 2, lower panels). Note that the normal growth temperature for yeast is 30 °C, and that 37 °C represents a mild heat shock, which would further challenge a yeast cell with impaired Hsp90 function. The V656M variation in HSP90β does not have any affect on yeast growth, even at elevated temperature (Fig. 2). Thus, this polymorphism does not cause any obvious dysfunction of the protein when assayed in yeast. These same plate growth assays confirm that Hsp90α can support growth, albeit not as well as authentic yeast Hsp90, and that changing the conserved residue Q488 produces a variant protein that functions even less efficiently. Yeast cells relying on Hsp90α Q488H grow more poorly, relative to those with the wild-type Hsp90α. Based on the comparison of the spots of the 10-fold serial dilution series, we estimate that Q488H supports growth 5- to 10-fold less well.

To determine if differences in Hsp90 protein expression could account for the slow growth phenotype, notably of Q488H, Hsp90 protein levels were analysed by immunoblotting. Human Hsp90 protein levels are compared in Fig. 3, before (left panels) and after loss (right panels) of yeast Hsp90. In both cases, there is no difference between the wild-type and variant versions of either Hsp90α or Hsp90β. The right panels also confirm that yeast Hsp90 was indeed lost from the strains with human Hsp90. Note that expression levels of Hsp90α and Hsp90β cannot be directly compared because isoform-specific antibodies were used. In any case, the functional defect of the Q488H variant of Hsp90α cannot be attributed to a difference in protein levels.

The Q488 polymorphism is located in the middle domain of Hsp90. This domain is the least well-characterized

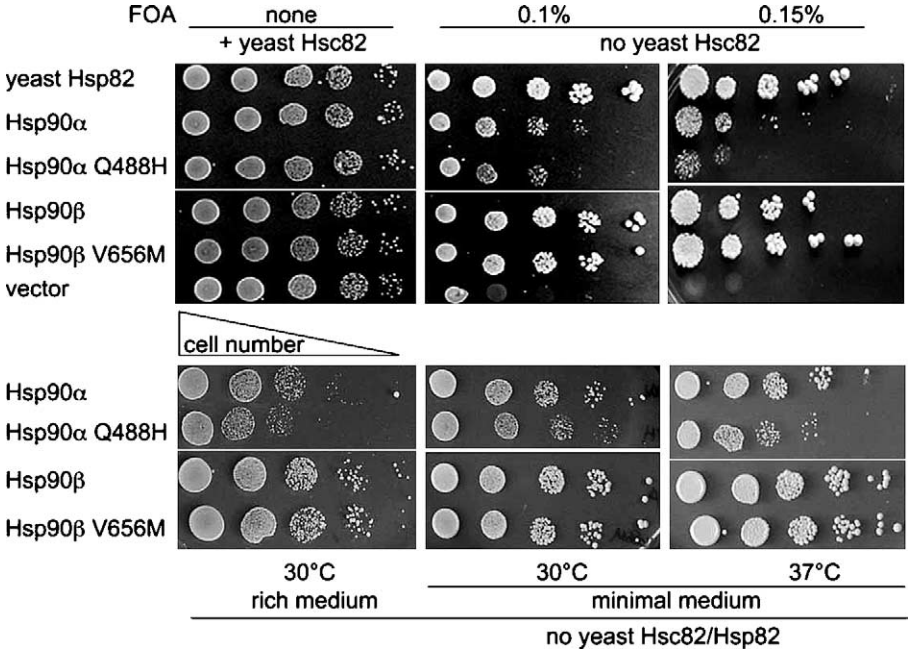

Fig. 2. Polymorphic variants of human Hsp90 support yeast growth to varying degrees. Top panel: cells expressing both yeast *HSC82* and a human *HSP90* as indicated from plasmids were grown to mid-exponential phase. Cultures were adjusted to the same cell density before serial dilutions were spotted onto solid media. Cells growing on plates containing FOA cannot have a functional *URA3* gene and therefore must have lost the Hsc82 expression vector. Note that all growth on FOA must be supported by heterologous expression of a human *HSP90* gene. Lower panel: FOA resistant cells retaining only a plasmid expressing human Hsp90 were isolated and cultured in media supplemented with uracil. Growth characteristics confered by expression of wild-type and variant human *HSP90* were determined by spotting serial dilutions onto minimal or rich media and incubation at 30 or 37 °C.

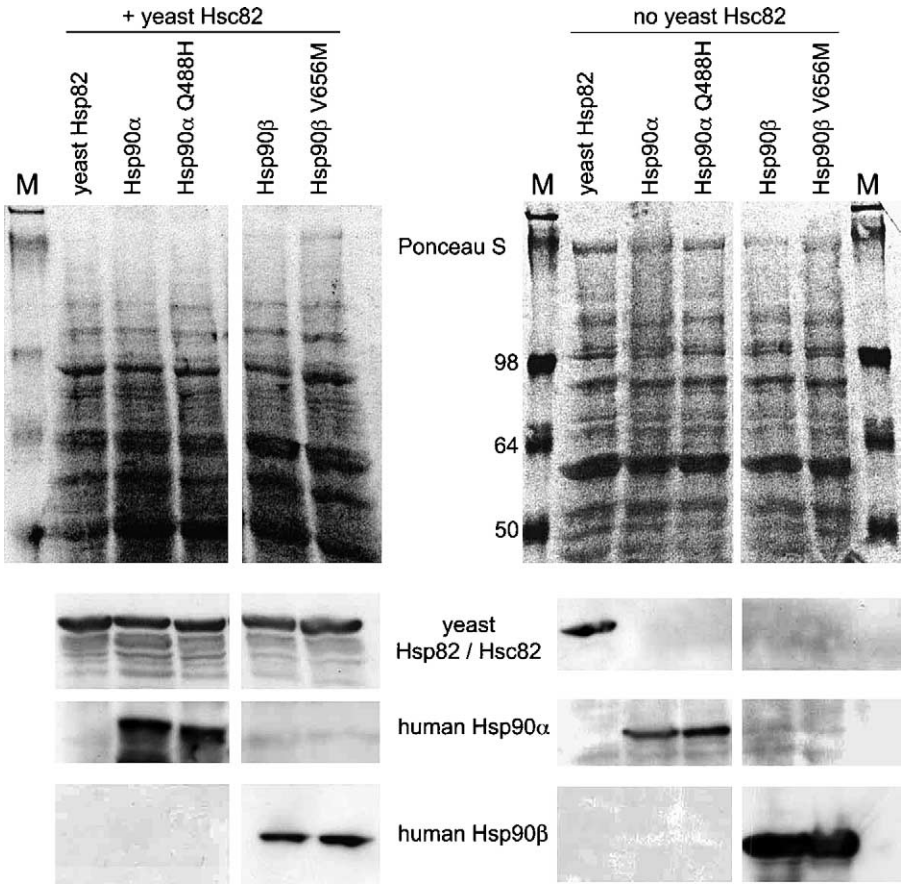

Fig. 3. Hsp90 protein expression. Expression of yeast or human Hsp90 was assessed by immunoblotting as described under Materials and methods. The numbers between the two top panels indicate the positions of molecular weight markers in kilodaltons.

portion of Hsp90. It has been proposed to be a major substrate binding site for substrate proteins as well as some Hsp90 co-chaperones [24,25]. It contains a catalytic loop that may contact the γ-phosphate of ATP bound to the N-terminal ATPase domain [24], thereby contributing to trapping ATP [26,27]. The Hsp90 co-chaperone Aha1 binds the middle domain and stimulates the ATPase activity of Hsp90, possibly by inducing the release of the catalytic loop [28]. However, most of the afore-mentioned interactions involve more N-terminal portions of the middle domain. Essentially nothing is known about the function of the sequences around Q488, although these regions may contribute to interdomain communications within Hsp90 [23,29]. Thus, this particular residue may not interact directly with co-chaperones or substrate proteins, but it could affect Hsp90 structure and function indirectly.

The individuals with these *HSP90* polymorphisms appeared to be healthy [9]. They presumably had only one "deviant" allele of either *HSP90α* or *HSP90β* and two wild-type alleles of the corresponding other gene. In yeast, neither of the two polymorphic Hsp90 proteins had any dominant negative effect. The phenotype of a homozygous *HSP90* polymorphism such as that encoding the defective Q488H variant is difficult to predict, but it is worth mentioning that the *hsp90β* knock-out in a *HSP90α* wild-type background has been found to be embryonically lethal in mice [30]. The *hsp90α* knock-out that would be relevant for the Q488H variant has not been reported. The effects of *HSP90* polymorphisms may only become apparent when an individual has a disease or ages. Because of the role of Hsp90 in chaperoning metastable substrate proteins, including many signalling proteins, it can also buffers the expression of genetic variation [4]. Thus, the response of an individual to environmental stress or diseases may depend on his/her Hsp90 status. Genetic variation may accumulate in genomes and remain phenotypically silent until Hsp90 function is challenged, even by modest environmental changes. Therefore, the polymorphism load and their effects merit further investigation to elucidate how different individuals would cope during ageing or stress when the level of misfolded and/or variant proteins increase.

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tion. We declare that all work carried out was in compliance with the current laws governing genetic experimentation in Switzerland.

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