Hsp90 is regulated by a switch point in the C-terminal domain

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Heat shock protein 90 (Hsp90) is an abundant, dimeric ATP-dependent molecular chaperone, and ATPase activity is essential for its *in vivo* functions. S-nitrosylation of a residue located in the carboxy-terminal domain has been shown to affect Hsp90 activity *in vivo*. To understand how variation of a specific amino acid far away from the amino-terminal ATP-binding site regulates Hsp90 functions, we mutated the corresponding residue and analysed yeast and human Hsp90 variants both *in vivo* and *in vitro*. Here, we show that this residue is a conserved, strong regulator of Hsp90 functions, including ATP hydrolysis and chaperone activity. Unexpectedly, the variants alter both the C-terminal and N-terminal association properties of Hsp90, and shift its conformational equilibrium within the ATPase cycle. Thus, S-nitrosylation of this residue allows the fast and efficient fine regulation of Hsp90.

Keywords: ATPase; chaperone; conformation; FRET; regulation

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INTRODUCTION

In eukaryotes, the molecular chaperone Heat shock protein 90 (Hsp90) modulates the conformation of metastable client proteins (Picard, 2002), including the tumor suppressor p53 (Muller *et al*, 2004; Walerych *et al*, 2004). The possibility to block the essential ATPase activity of Hsp90 (Obermann *et al*, 1998; Panaretou *et al*, 1998) through specific inhibitors makes Hsp90 an interesting target for anti-cancer therapy (Whitesell & Lindquist, 2005). Hsp90 is a dimeric protein with an amino-terminal nucleotide binding pocket, a middle domain known to act as a discriminator between Hsp90 clients (Hawle *et al*, 2006) and a carboxy-terminal dimerization domain (Fig 1A). The mechanism of ATP hydrolysis

is conserved for various Hsp90 family members (Ali *et al*, 2006; Dollins *et al*, 2007; Frey *et al*, 2007; Graf *et al*, 2009; Hessling *et al*, 2009; Leskovaar *et al*, 2008; Richter *et al*, 2008; Shiao *et al*, 2006). During the ATPase cycle, Hsp90 passes through several conformations, including an open state and an ATP-bound closed state, stabilized by a transient N-terminal dimerization (Ali *et al*, 2006; Richter *et al*, 2006; Wandinger *et al*, 2008). The C-terminal dimerization domain of yeast Hsp90 (yHsp90) does not seem to contribute directly as it can be substituted by a disulphide bridge (Wegele *et al*, 2003).

The ATPase cycle is modulated by co-chaperones such as the stimulator Aha1 (Meyer *et al*, 2004; Panaretou *et al*, 2002) or the inhibitors Hop/Sti1 (Prodromou *et al*, 1999; Richter *et al*, 2003) and p23/Sba1 (Forafonov *et al*, 2008; Johnson *et al*, 1994; Richter *et al*, 2004; Prodromou *et al*, 2000; Young & Hartl, 2000). A further level of regulation are post-translational modifications of Hsp90 such as phosphorylation, acetylation (Scroggins *et al*, 2007; Wandinger *et al*, 2006) and S-nitrosylation, the modification of the cysteine residues of Hsp90 by nitric oxide (NO; Jorge *et al*, 2007; Rhee *et al*, 2005; Lindermayr *et al*, 2005). In human Hsp90 α (hHsp90 α), S-nitrosylation occurs at Cys 597 (Martinez-Ruiz *et al*, 2005); notably, this residue is located in the C-terminal domain of Hsp90. S-nitrosylation affects the Hsp90 ATPase activity and binding of endothelial nitric oxide synthase to Hsp90 (Garcia-Cardena *et al*, 1998; Martinez-Ruiz *et al*, 2005). These results suggest that the C-terminal domain has a, so far, unknown function in the modulation of Hsp90 activity.

RESULTS

A conserved cysteine residue in the Hsp90 family

Hsp90 has a highly conserved domain structure (Fig 1A). Several Hsp90 family members have been shown to be S-nitrosylated (Cys598; Fig 1B; Martinez-Ruiz *et al*, 2005; Jorge *et al*, 2007; Rhee *et al*, 2005; Lindermayr *et al*, 2005). The NO-modifiable cysteine residue is conserved in eukaryotes and in some bacteria (Fig 1C), whereas, in other Hsp90 species it has been replaced (Fig 1D). A surface representation of the yHsp90 dimer shows that the corresponding residue in yHsp90 (Ala577) is part of a three-strand β -sheet (Fig 1E). On the basis of its location, a direct impact on the yHsp90 ATPase activity was not expected. To test

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Fig 1 | Sequence alignment of Heat shock protein 90 homologues and structure of the Hsp90 carboxy-terminal domain. (A) Schematic domain organization of yeast Heat shock protein 90 (yHsp90). Ala 577 is shown in red. (B–D) Alignment of Hsp90 family members. (B) For these Hsp90 proteins, a proteomic-based analysis revealed cysteine modification of Hsp90 by nitric oxide (Martinez-Ruiz *et al*, 2005; Jorge *et al*, 2007, Rhee *et al*, 2005; Lindermayr *et al*, 2005). In human Hsp90 α (hHsp90 α), Cys 597 was found to be S-nitrosylated. (C) Alignment of representatives of Hsp90 showing a conserved cysteine residue with the respective position highlighted in red. (D) Hsp90 family members lacking the conserved cysteine residue. (E) Surface presentation of yHsp90 in the closed conformation (PDB code: 2CG9). The position marked using a square indicates Ala 577, which corresponds to Cys 597 in hHsp90 α . The enlargement (right) shows Ala 577 in red and hydrogen bonds as black dotted lines.

whether this residue regulates Hsp90 function in general, we characterized mutants for this position in both yHsp90 and hHsp90.

ATPase activity of Hsp90 variants and S-nitrosylated Hsp90

An inhibitory effect of the ATPase activity of hHsp90 α following NO treatment has been reported previously (Martinez-Ruiz *et al*, 2005). We tested several yHsp90 mutants at the corresponding position (Ala 577) for their intrinsic ATPase activity (Fig 2A). Surprisingly, the ATPase activity was enhanced by a substitution with cysteine or isoleucine (fourfold) or reduced by a substitution

with asparagine or aspartic acid (twofold), with a more pronounced effect at lower temperatures (supplementary Table S1 online). Notably, when yHsp90A577C was S-nitrosylated, the ATPase activity was reduced to approximately 50% (Fig 2B). To address the regional specificity of the effect, structurally related positions in yHsp90 were mutated: the neighbouring Ala 576 in the same β -sheet strand, Ala 509 in an α -helix and Pro 486 located in close spatial proximity to position 577. None of these yHsp90 mutants affected the ATPase to the extent observed for mutants of position 577 (supplementary Table S1 online). For the ATPase

Fig 2 | Carboxy-terminal modifications alter Heat shock protein 90 ATPase activity. (A) Steady state k_{cat} values of wild-type yeast Heat shock protein 90 (yHSP90) and yHsp90A577X mutants. (B) yHsp90 was treated with NO *in vitro* as described. The efficiency for S-nitrosylation of the yHsp90A577C mutant was 73%, as determined using an Ellman assay. (C–F) Stimulating effect of the Hsp90 co-chaperone Aha1 on the ATPase activity of Hsp90 variants. (C) Steady state k_{cat} values of wild-type yHsp90 (open circles), yHsp90A577C (open squares), yHsp90A577C modified with NO (filled squares) and yHsp90A577N (open triangles) using 1 μ M of yHsp90 and increasing amounts of Aha1. Data were fitted according to equation (1); see supplementary information online. (D–F) Steady state $k_{cat,max}$ values at Aha1 saturation for variants of yHsp90 (D), human HSP90 β (hHsp90 β) (E) and hHsp90 α (F). The inset in (E) represents the stimulation of wild-type hHsp90 β (open circles), wild-type hHsp90 β modified with NO (filled circles) and hHsp90C590A (open triangles). The inset in (F) represents stimulation of wild-type hHsp90 α (open circles), wild-type hHsp90 α modified with NO (filled circles) and hHsp90 α C598A (open triangles). Experiments in (C–F) were measured at 37 °C in 40 mM HEPES, pH 7.5, and 20 mM KCl. NO, nitric oxide; wt, wild type.

activity of the two human isoforms, we observed, against expectation, only little influence of both S-nitrosylation and substitution of the reactive cysteine residue against other amino acids (supplementary Fig S1 online). As the low intrinsic hHsp90 ATPase activity makes it difficult to detect changes that further decrease the ATPase rate, we used the well-characterized Aha1-mediated stimulation of Hsp90 (Richter *et al*, 2008) for further analysis. In the presence of Aha1, the activity of NO-modified yHsp90A577C was one-third of that of the untreated yHsp90A577C (Fig 2C). Furthermore, yHsp90A577N and yHsp90A577D showed a reduction in the maximum Aha1-stimulation compared with wild-type yHsp90 (Fig 2C,D). In addition, the affinities for Aha1 binding decreased significantly after NO treatment of yHsp90A577C compared with untreated yHsp90A577C ($K_{D,app} \approx 4.2 \mu$ M versus $K_{D,app} \approx 1.6 \mu$ M). For the mimicking mutation yHsp90A577N, $K_{D,app}$ decreased tenfold compared with the wild-type protein ($K_{D,app} \approx 32 \mu$ M versus $K_{D,app} \approx 3.1 \mu$ M). The influence of Aha1 on

the Hsp90 ATPase activity was highly specific, whereas for the other co-chaperones tested, such as Cpr6, p23/Sba1 and Hop/Sti1, no differences in the regulation of the ATPase activity of yHsp90 and its variants were detected (supplementary Fig S2 online). Interestingly, Aha1 also affected the hHsp90 variants differentially (Fig 2E,F). Here, NO treatment of the hHsp90 wild-type proteins, and the S-nitrosylation-mimicking mutations in hHsp90 β and hHsp90 α showed a pronounced reduction in Aha1-stimulated ATPase activity. The binding affinity of Aha1 to hHsp90 and the maximum stimulation efficiency is significantly reduced by NO treatment and S-nitrosylation-mimicking mutations, comparable with the yHsp90 variants.

Residue 577 affects the dimerization of yHsp 90

The effects observed so far point towards a crucial regulatory function for position 577. However, no structural change was observed when the yHsp90 wild-type and mutants proteins were

Fig 3 | Effects of Heat shock protein 90 mutations on carboxy- and amino-terminal dimerization determined using FRET. (A) Subunit exchange of different yeast Heat shock protein 90 (yHsp90) dimers. The decrease in the fluorescein signal was monitored ($\lambda_{\text{ex}} = 496 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$) after mixing 100 nM FITC-labelled yHsp90 with 100 nM TAMRA-labelled yHsp90. The data were corrected for linear bleaching. Wild-type yHsp90, blue; yHsp90A577I, orange; and yHsp90A577N, green. ATPase activities for mutants are given in supplementary Table S2 online. (B) Amino-terminal dimerization of the preformed FRET pairs of yHsp90 variants after the addition of 2 mM ATP γ S, monitored by following the decrease in the fluorescein signal as in (A). The colour code is the same as in (A). FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; TAMRA, tetramethyl-6-carboxyrhodamine; wt, wild-type.

analysed by circular dichroism spectroscopy or nuclear magnetic resonance spectroscopy (supplementary Fig S3, S4A,B online). To test whether the mutations might have an effect on the dimerization properties of Hsp90, we first showed that the yHsp90A577C mutant forms heterodimers with the ATPase-deficient mutant $\Delta 24$ Hsp90 (supplementary Fig S5 online), similar to wild-type yHsp90 (Richter *et al*, 2001). Then, we monitored the kinetics of dimerization between donor- and acceptor-labelled yHsp90 variants, by using fluorescence resonance energy transfer (FRET; Hessling *et al*, 2009; Fig 3A; supplementary Table S2 online). Interestingly, for the yHsp90 mutant with a high ATPase activity (yHsp90A577I), the apparent half-life for subunit exchange was enhanced fivefold compared with the wild-type yHsp90 (Table 1). By contrast, the yHsp90A577N mutant with almost no ATPase activity showed a twofold reduced half-life of subunit exchange (Table 1) and a reduced FRET efficiency (Fig 3A). These effects are in line with the apparent K_D values observed for the C-terminal association of yHsp90 monitored by size exclusion high performance liquid chromatography. Here, the yHsp90A577I mutant showed a stronger association compared with wild-type or yHsp90A577N (Table 1).

The transient association of the N-terminal domains, monitored using FRET after the addition of ATP γ S (Hessling *et al*, 2009), showed only minimal differences for wild-type yHsp90 compared with yHsp90A577I (Fig 3B). For the yHsp90A577N mutant, however, the N-terminal association after nucleotide binding was strongly impaired (Table 1) and the association properties were changed (Fig 3B).

Effect of the Hsp90 mutants on client protein processing

To test whether the Hsp90 variants described above affect client protein activation, we used hepatitis-B virus reverse transcriptase (RT) as an authentic Hsp90 client. Recently, it has been shown that hHsp90 and yHsp90 activate RT in the presence of

Hsc70/Hsp40 and co-chaperones (Beck & Nassal, 2003; Stahl *et al*, 2007). All yHsp90 variants used in this study supported the RT priming activity, although with different efficiencies (Fig 4A). RT activation was stimulated more strongly by yHsp90A577C and yHsp90A577I compared with wild-type yHsp90. The most pronounced decrease was observed for yHsp90A577N and yHsp90A577D compared with wild-type yHsp90 (Fig 4A), which is comparable with the effect of NO modified yHsp90A577C (Fig 4B). This strict correlation between chaperone activity and mutation was also detected for hHsp90 β (Fig 4C) and hHsp90 α variants (Fig 4D). Here again, the treatment of hHsp90 β or hHsp90 α with NO showed comparable effects on the activity of Hsp90 as the alanine, asparagine or aspartic acid mutations.

In vivo analysis of yHsp90 mutants

To characterize the Hsp90 variants *in vivo*, a *Saccharomyces cerevisiae* strain was analysed in which plasmid-encoded HSP82 variants were the only source of Hsp90. All yHsp90 mutants supported the functions essential for viability at 30 °C and showed nearly the same temperature phenotype until a maximum growth temperature of approximately 39 °C (supplementary Fig S6A online). To analyse the Hsp90-mediated client protein activation *in vivo*, v-Src, which is strictly dependent on fully active Hsp90 (Nathan & Lindquist, 1995), was co-transfected and tested for activity (Fig 5A, inset). The expression levels of yHsp90 variants were identical in these experiments (supplementary Fig S6B online). Markedly, we found that *in vivo* the mutants with higher intrinsic ATPase activity showed higher client activation and *vice versa* (Fig 5A). The yHsp90A577I mutant stimulated v-Src activity to about 170% and yHsp90A577D to about 40% compared with wild-type yHsp90. When human Hsp90 variants were expressed in this deletion strain, a similar picture emerged. Both alanine mutants of hHsp90 β and hHsp90 α showed a significant decrease in v-Src activity compared with wild-type

Table 1 | Effects of yeast Heat shock protein 90 variants on carboxy-terminal and amino-terminal dimerization

yHsp90 variants	$t_{1/2,app}$ subunit exchange (min ⁻¹)*	$t_{1/2,app}$ N-terminal dimerization (min ⁻¹)*	app. K_D Hsp90 _{C-terminus} (nM ⁻¹)	k_{cat} ATPase at 25 °C (min ⁻¹)
Wild type	1.02 ± 0.02	9.42 ± 0.07	34 ± 5	0.20 ± 0.03
A577I	5.19 ± 0.03	6.73 ± 0.08	25 ± 2	1.43 ± 0.03
A577N	0.62 ± 0.02	25.65 ± 1.25	71 ± 6	0.01 ± 0.01

The apparent half-life is derived from the fluorescence resonance energy transfer (FRET) experiments for the subunit exchange ($t_{1/2,app}$ subunit exchange), shown in Fig 3A, and for the nucleotide-induced amino terminal association ($t_{1/2,app}$ N-terminal dimerization), shown in Fig 3B. The qualitative half-life was determined as described in the supplementary information online, equation (2), including s.d. For determination of $t_{1/2,app}$ using FRET, the cysteine mutants of Hsp90 were used, as indicated by an asterisk. The apparent K_D values for yHsp90 wild type, yHsp90A577I and yHsp90A577N were determined using size exclusion high performance liquid chromatography as described in the supplementary information online. The listed k_{cat} values for yHsp90 wild type and mutants are taken from supplementary Table S1 online for better comparison.

Fig 4 | Effect of Heat shock protein 90 nitric oxide modification and Hsp90 mutations on reverse transcriptase activation. (A–D) Priming competent RT–D-ε-RNA complexes were reconstituted as described in the supplementary information online. The ³²P-labelled RT was visualized by autoradiography, as shown in the Fig 4A inset. (A) Densitometric analysis of the effects of yeast Heat shock protein 90 (yHsp90) wild-type and mutants on the formation of the priming-active RT–D-ε-RNA complex. (B) Influence of NO on RT–D-ε-RNA complex formation. (C–D) Effects of human HSP90β (hHSP90β) variants (C) and hHsp90α variants (D) on RT–D-ε-RNA complex formation and the influence of NO on hHsp90 activity. Experiments were conducted as in (A) and (B). All experiments were carried out in triplicates; error bars indicate s.d. NO, nitric oxide; RT, reverse transcriptase; wt, wild-type.

proteins (Fig 5B). Thus, client protein activation is affected by the human and yeast Hsp90 variants similarly *in vitro* and *in vivo*.

DISCUSSION

The S-nitrosylation of Hsp90 affects one cysteine residue in the C-terminal domain of hHsp90α (Martinez-Ruiz *et al*, 2005), which

is part of a secondary structure element. *In silico*-based analysis suggests that this residue is involved in regulating the conformation in Hsp90 (Morra *et al*, 2009). By variation of this particular residue in yHsp90 and hHsp90, we could modulate their activities and mimic the S-nitrosylated form. Interestingly, the impact of the introduced amino acid on Hsp90 activity is in line with the

Fig 5 | *In vivo* chaperone effect of Heat shock protein 90 mutants. (A) A yeast *HSC/HSP82*^{-/-} deletion strain was transfected with *HSP82* wild-type (wt) or single point mutants and a plasmid containing *V-SRC*. The phosphorylation activity of v-Src was analysed by Western blotting using a phosphotyrosine antibody (4G10), as shown in Fig 5A inset (see also supplementary Fig S6B online). The average of three independent experiments was analysed using densitometry. (B) The yeast deletion strain was transfected with human Heat shock protein 90β (*hHSP90β*) and *hHSP90α* wild-type or the alanine mutations, co-transfected with *V-SRC* and analysed as in (A). The error bars indicate s.d.

propensity of the respective amino acid to stabilize β-sheets (Minor & Kim, 1994); for example, substitution with asparagine, a β-sheet destabilizer, resulted in a decrease in Hsp90 activity. Our results suggest that the dynamic equilibrium of Hsp90 is influenced by affecting the C-terminal and the nucleotide-dependent N-terminal dimerization. We speculate that NO modification, as well as the substitution of the respective amino acid with *S*-nitrosylation-mimicking residues such as asparagine and aspartic acid, shifts the conformational equilibrium of Hsp90 towards the open conformation. This increases the energy barrier necessary for conformational changes, leading to N-terminal dimerization and consequently to a less active chaperone. The switch point seems to be conserved in the Hsp90 family. The

appearance of an NO-modifiable cysteine residue in Hsp90 in higher eukaryotes allows the fine-tuning of Hsp90 activity by reversible post-translational modification.

Taken together, our results highlight the importance of a single crucial residue in the C-terminal domain as a switch point in the pathway of conformational transitions in Hsp90, and as a regulator of the domain communications in the Hsp90 dimer that consequently affects substrate activation.

METHODS

Protein expression and modifications. All constructs were cloned in the vector pET28a (Invitrogen, Karlsruhe, Germany), expressed in *Escherichia coli* strain BL21 (DE3) cod⁺ (Stratagene, La Jolla, CA, USA) and the proteins were purified as described previously (Richter *et al*, 2001, 2008). For *S*-nitrosylation of Hsp90, a 20-fold excess of the NO donor diethylamine NONOate sodium salt hydrate ((CH₃CH₂)₂N-N(N=O)O⁻Na⁺·xH₂O; Sigma, Munich, Germany) was incubated with Hsp90 for 30 min at 30 °C and the excess label was separated using a desalting column (HiTrap Desalting column; GE Bioscience Europe, Freiburg, Germany).

Determination of ATPase activity. ATPase assays were carried out as described previously, using an ATP-regenerating system (Ali *et al*, 1993; Panaretou *et al*, 1998). For subtraction of background ATPase activity, the assay was stopped by the addition of 125 μM Radicol (Sigma, Munich, Germany) and the ATPase activity was calculated using linear regression.

Subunit exchange and N-terminal dimerization of yHsp90 mutants visualized by FRET. Double mutants containing a cysteine residue and either yHsp90A577N or yHsp90A577I were created and labelled for FRET experiments. For subunit exchange, 100 nM of fluorescein isothiocyanate (FITC)-labelled yHsp90 was incubated at 25 °C. After the addition of 100 nM tetramethyl-6-carboxyrhodamine-labelled yHsp90, the decrease in the FITC signal was recorded (λ_{ex} = 496 nm; λ_{em} = 520 nm).

N-terminal dimerization. A total of 2 mM ATPγS (Roche, Mannheim, Germany) was added to the Hsp90 heterodimers and the decrease in FITC signal was recorded (see supplementary information online).

Reconstitution of RT-D-ε-RNA complexes and priming assay. Reconstitution of hepatitis-B virus RT in complex with its D-ε-RNA was carried out as described previously (Stahl *et al*, 2007). In brief, standard reconstitution reactions contained a fusion protein of RT, Hsc70, Hsp40, Hop and Hsp90 wild-type, mutants thereof and NO-treated proteins, an ATP-regenerating system, RNasin (RNase inhibitor; Promega, Mannheim, Germany), *in vitro* transcribed D-ε-RNA and H₂O. DNA synthesis activity was monitored by the priming assay described by Beck & Nassal (2003).

The v-Src kinase assay. A *S. cerevisiae* strain, carrying an *HSC82/HSP82* double knockout mutation (Nathan & Lindquist, 1995), was transfected with *HSP90* variants, cloned in the high-copy vector pRS423 (2 μ) and co-transfected with *V-SRC* present on a vector carrying a *URA3* marker. The v-Src assay was determined as the total cellular amount of tyrosine-phosphorylated proteins (Nathan & Lindquist, 1995) using the specific antibody 4G10 (Stratagene, La Jolla, CA, USA).

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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