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Middle domain of human Hsp90 isoforms differentially binds Aha1 in human cells and alters Hsp90 activity in yeast

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ABSTRACT

Hsp90 is an essential chaperone for more than 200 client proteins in eukaryotic cells. The human genome encodes two highly similar cytosolic Hsp90 proteins called Hsp90α and Hsp90β. Most of the client proteins can interact with either Hsp90 protein; however, only a handful client proteins and one co-chaperone that interact specifically with one of the Hsp90 isoforms were identified. Structural differences underlying these isoform-specific interactions were not studied. Here we report for the first time that the Hsp90 co-chaperone Aha1 interacts preferentially with Hsp90α. The distinction depends on the middle domain of Hsp90. The middle domain of Hsp90α is also responsible for the slow growth phenotype of yeasts that express this isoform as a sole source of Hsp90. These results suggest that differences in the middle domain of Hsp90α and Hsp90β may be responsible for the isoform-specific interactions with selected proteins. Also shown here within, we determine that preferential chaperoning of cIAP1 by Hsp90β is mediated by the N-terminal domain of this isoform.

1. Introduction

The 90-kDa heat shock protein (Hsp90) is an essential chaperone in eukaryotic cells [1]. Hsp90 structure is composed of three distinct domains: N-terminal ATPase domain (N-domain), middle domain (M-domain) that contains the major site of the interaction with client proteins, and C-terminal domain (C-domain) responsible for Hsp90 dimerization [2–5].

In the human genome, there are two genes that encode the 86% identical cytoplasmic proteins (isoforms) called Hsp90α and Hsp90β (also named Hsp90AA1 and Hsp90AB1). Together, these isoforms constitute up to 2% of total cellular protein [6], but the ratio between the isoforms varies significantly in different tissues and cell lines [7,8]. Most tissues express both isoforms, but Hsp90β is usually more abundant, except for the brain and the oocytes [9,10]. Hsp90α is thought to be more inducible than Hsp90β under stress conditions [11,12]. However, in human kidney cells, transcription of both isoforms can be equally induced under stress [13]. In solution, Hsp90α exists as a dimer, whereas Hsp90β can be found both as a dimer and monomer [14,15]. Despite high sequence identity, no heterodimers of the isoforms were observed [16].

Hsp90 interacts with more than 200 client proteins [17,18]. Most of these proteins are thought to be chaperoned by both Hsp90 isoforms. Relatively few examples, listed below, of a preferential interaction of a client protein with one Hsp90 isoform or isoform-specific function were reported. Hsp90α, for example, is indispensable for the antigen processing necessary to generate peptide-loaded MHC I complexes, for spermatocyte maturation and for maturation of the hERG ion channel [19–21]. Hsp90α can be secreted to the extracellular matrix, where it plays a vital role in cancer metastasis by regulating metalloproteinase activity [22]. Mouse knockout studies demonstrated that Hsp90β is required for the proper formation of placenta [23]. Several proteins such as cIAP1 and vitamin D receptor were shown to interact preferentially with Hsp90β [24,25]. Hsp90β is also involved in the response of macrophages and dendritic cells to bacterial DNA [26]. It was also reported that Hsp90α and Hsp90β have opposing effects on endothelial nitric oxide synthase (eNOS) activity. Nitric oxide production by eNOS is stimulated by overexpression of Hsp90α but inhibited by Hsp90β, a property that was attributed to the higher stability of Hsp90α dimers [27]. Hsp90α and Hsp90β have also the opposite effect of on the biogenesis of KCNQ4 channels [28].

Hsp90 function is regulated by numerous co-chaperone proteins. Co-chaperones help Hsp90 to form complexes with client proteins and regulate Hsp90 ATPase activity [29]. The only known example of an isoform-specific co-chaperone is Gcunc45 that interacts preferentially with the Hsp90β [30]. A complete list of Hsp90 interacting proteins with references can be found at www.picard.ch/downloads.html

No studies on the structural features of human Hsp90s underlying isoform specificity have been reported to date. Therefore, we decided to investigate which fragments of human Hsp90 may be responsible

Abbreviations: 17-AAG, 17-Allylamino-17-demethoxygeldanamycin; aa, amino acid; cIAP1, cellular inhibitor of apoptosis protein-1

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for the differing interactions of Hsp90α and Hsp90β with co-chaperone Aha1 and the client protein cIAP1.

2. Methods

2.1. Plasmids construction

Plasmids for the expression of human Hsp90 α and β genes in yeast and plasmids for expression in human cells that contain Hsp90 genes fused to the N-terminal Flag-HA tags were described previously [31]. These plasmids served as templates in the PCR reactions to create fragments used to construct hybrid Hsp90 genes containing fragments of the Hsp90α and Hsp90β isoforms. Hsp90 hybrids were constructed by a two-step PCR. Fragments of the Hsp90α and Hsp90β were amplified using primers that created 30–40 bp overlaps at the junction site. These fragments served as templates in an overlap extension PCR with primers that added 25 bp sequences homologous to the polylinkers of the cloning vectors [32]. Hybrid Hsp90 used to create yeast expression vectors were created using wild-type Hsp90α and Hsp90β sequences. Hybrids used in experiments in human cells were made of genes that contained a single amino acid substitution, Hsp90α $Ile_{128}Thr$ and Hsp90β $Ile_{123}Thr$.

Construction of the plasmid p423TDH3 was described previously [31]. Hsp90 genes for expression in yeast were cloned into plasmid p423TDH3 digested with enzymes *Spe*I and *Sma*I. Plasmids used for human cell transfection were made by cloning of the Hsp90 genes in the plasmid pcDNA3.1 digested with enzymes *Kpn*I and *Xho*I. The final PCR products containing complete hybrid Hsp90 genes were inserted into vectors digested with the indicated above restriction enzymes using cohesive ends generated with T4-polymerase as described earlier [33].

Details on the created Hsp90 hybrids are provided in Fig. 1 and Supplementary Fig. 1.

The open reading frame of the human cIAP1 was cloned in the plasmid pcDNA3.1. Point mutations in the Hsp90 were generated by PCR with mutagenic primers [32]. Oligonucleotides used in this study are listed in Supplementary Table 1. Hsp90 alleles with $Ile_{128}Thr$ and $Ile_{123}Thr$ mutations, which were used in experiments with HEK-293 cells, were marked in the text with superscript IT, to distinguish them from the wild-type alleles used in experiments in yeasts.

2.2. Yeast strains and yeast growth assays

Saccharomyces cerevisiae strain used in this study had genotype ade2 ura3 trp1 leu2 his3 lys2 MATa hsc82::G418 Hsp82::G418. Both endogenous Hsp90 genes were replaced in this strain with G418 markers. The strain carried the plasmid with Ura3-selective marker and human Hsp90α gene expressed from the TDH3 promoter. The construction of this strain was described previously [31]. This strain was transformed with plasmids containing different Hsp90 genes and a His3 marker. The obtained $HIS3^+$ transformants were selected for loss of Ura3-based plasmid in medium containing 0.1% 5' fluoroorotic acid [34]. The constructed strains that expressed human Hsp90α and Hsp90β were named BY561 and BY562, respectively.

Strains BY561 and BY562 were used to construct mutants in which *AHA1* and *HCH1* open reading frames were replaced with TRP1 gene. DNA fragments used for the gene replacement were amplified by PCR with primers listed in Supplementary Table 1 using plasmid pRS414 as a template. Yeasts were transformed with these PCR products, and transformants were selected on plates with medium lacking tryptophan. Successful replacement of *AHA1* and *HCH1* coding sequences with TRP1 marker was confirmed by PCR with test primers listed in Supplementary Table 1.

For the growth assays, yeast strains were cultured in YPD medium supplemented with 100 mg/l of adenine and uracil. The density of the saturated cultures was adjusted to equal OD_{600} and a series of dilutions made from the adjusted cultured were spotted on YPD-agar plates incubated at 30 °C. The growth of BY561 and BY562 strains and the strains with additional disruptions of *AHA1* and *HCH1* genes were monitored

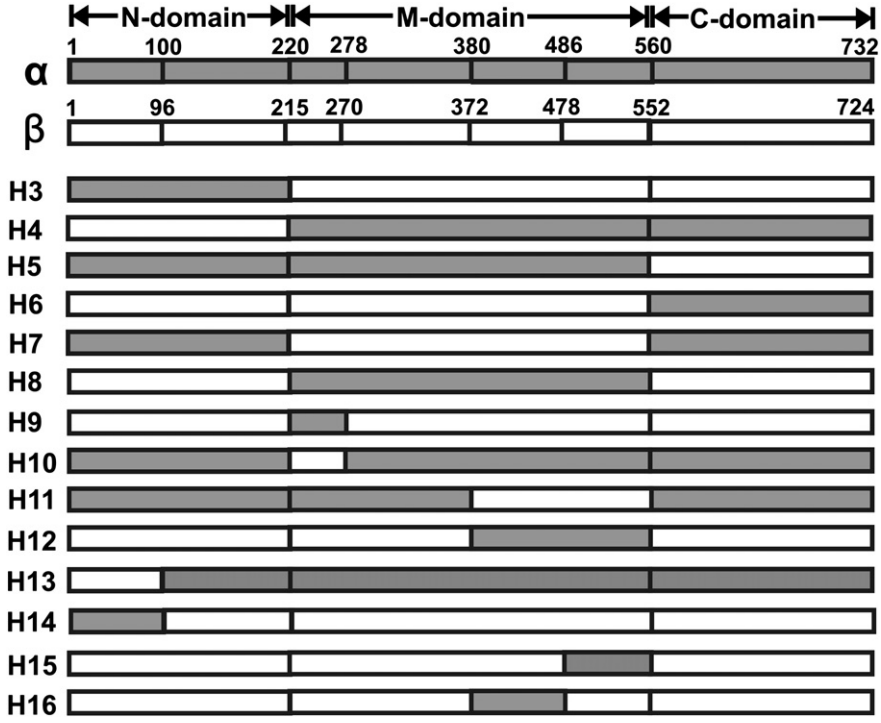

Fig. 1. Diagram of Hsp90 hybrid genes used in this work. Sequences of Hsp90α labeled in gray, sequences of Hsp90β labeled in white. Numbers indicate amino acid residues at the junctions of the fragments used to create hybrid sequences.

using Bioscreen C microplate reader (Oy Growth Curves Ab Ltd) in liquid YPD supplemented with 100 mg/l of adenine and uracil at 30 °C.

2.3. Culture and transfection of human cells

HEK-293 cells were cultured in Iscove's modified Dulbecco's medium (IMDM). Cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions (Life Technology). After transfection with 17-AAG-resistant Hsp90 variants (mutants $Ile_{128}Thr$ in Hsp90α N-domain and corresponding $Ile_{123}Thr$ in Hsp90β N-domain), cells that express high level of transfected Hsp90 proteins were selected in IMDM medium supplemented with 1 μM 17-AAG, 10% calf serum, and a standard penicillin–streptomycin solution. During selection, cells were trypsinized and passaged to fresh culture plates in 1 μM 17-AAG every 2–3 days. Control cells transfected with the pcDNA3.1 vector were treated identically to monitor the process of selection.

2.4. Immunoprecipitations

Immunoprecipitation of Flag-tagged Hsp90

For immunoprecipitation, cells were grown to about 70% confluency in 10 cm culture dishes. Immunoprecipitation was performed using anti-Flag antibodies covalently linked to agarose beads (Sigma-Aldrich). Cells were lysed at 70–90% confluency in 1 ml of buffer containing 25 mM Tris pH 7.6, 1 mM ATP, 0.5% Triton X-100, and protease and phosphatase cocktails (Roche). Insoluble debris was removed by centrifugation for 15 min at 14 000 rpm. Cleared lysate was incubated with antibody-coupled beads (20 μl volume) at 4 °C for 2 h with constant mixing. Beads were washed three times with 25 mM Tris pH 7.6. Bound proteins were eluted with 1% SDS and analyzed by western blot.

Immunoprecipitation of wild-type Hsp90

Seventy percent confluent HEK-293 cells cultured in 10 cm plates were transfected with N-terminally tagged with FLAG sequence Hsp90α and Hsp90β genes cloned in pcDNA3.1 plasmid [31]. Cells were cultured in IMDM medium supplemented with 10% serum and standard penicillin–streptomycin solution, without 17-AAG for 48 h before immunoprecipitation experiment. Lysis was performed in 1 ml of lysis buffer described above except for ATP that was replaced with 5 mM non-hydrolyzable ATP analog 5′-adenylylimidodiphosphate (AMPPnP). Immunoprecipitation was performed as described above.

2.5. Chaperoning activity of hybrid Hsp90s for cIAP-1

HEK-293 cells that stably expressed Hsp90 17-AAG-resistant variants of hybrid Hsp90 proteins were transfected with the cIAP1 gene fused to c-terminal Myc-tag cloned in pcDNA3.1 plasmid using GeneCellin according to the manufacturer's instructions (BioCellChallenge). Transfected cells were cultured in medium without antibiotics. Twenty-four hours after transfection, the cells were harvested, lysed in buffer containing 25 mM Tris, pH 7.6, 0.5% Triton X-100, and cocktails of protease and phosphatase inhibitors (Roche). Lysates were centrifuged at 14000 rpm for 10 min. Protein concentration was measured using BCA assay (Pierce). Equal amounts of protein for each sample were loaded on the gel.

The antibodies used for western blot analysis were as follows: FLAG (Sigma-Aldrich, F3165), Aha1 (Abcam, ab56721), Cdc37 (Abcam, ab109419), Hsp90β (Abcam, ab119833), actin (MP Biomedicals #69100), and Myc-Tag (Cell Signaling, 71D10)

3. Results

3.1. Analysis of the hybrid human Hsp90s activity in yeasts

Each of the human Hsp90 can replace both yeast homologues, but cells that express only Hsp90α grow much slower than cells that express only Hsp90β [35]. We reasoned that this difference may be used to identify structural differences in the human Hsp90 isoforms. Hence, we created a number of yeast strains that expressed point mutants of human Hsp90 or Hsp90 hybrids composed of fragments of human Hsp90α and Hsp90β as a sole form of this protein. Growth rate of these strains was correlated with the structural features of the expressed Hsp90 proteins.

3.2. Dimerization is not a major factor in isoform differences

Hsp90 chaperone acts as a homodimer. Dimers formed by the Hsp90α are more stable than dimers of Hsp90β, which may influence the activity of the isoforms. Therefore, we decided to test whether activity of the human Hsp90 isoforms in yeasts correlates with their ability to dimerize. Substitution of alanine 629 with methionine decreases dimerization of Hsp90α, whereas mutation $Met_{621}Ala$ in Hsp90β stabilizes dimers of this isoform [36]. We constructed yeast strains that expressed Hsp90 with these amino acid (aa) substitutions and compared their growth with that of the control strains that expressed wild-type human Hsp90α or Hsp90β. Results presented in Fig. 2A demonstrate that growth rates of the mutant strains were similar to those of controls, indicating that stability of the dimers is not a major factor in different ability of the isoforms to support growth of yeasts.

3.3. Differences in Hsp90 isoforms activity are caused by differences in the M-domain structure

We hypothesized that growth phenotype of the yeasts that express human Hsp90 depends on structural differences located in particular domain of this protein. To test this hypothesis, we constructed a number of hybrid Hsp90 genes in which the fragments that encode N-, M-, or C-domains of the Hsp90α sequence were substituted with the corresponding sequences of Hsp90β (Fig. 1). We created also hybrids H9 and H10 in which charged linker regions (aa 220-278 in Hsp90α and aa 215-270 in Hsp90β), the most divergent fragments of the human Hsp90α/β, were swapped. The hybrids H13 and H14 with swapped sequences of the N-terminal ends were also constructed because of the involvement of these sequences in the N-domain dimerization essential for Hsp90 activity [37]. Plasmids carrying these hybrids were transformed into yeast cells to replace an original plasmid with the human Hsp90α. Yeasts expressing hybrids with only a small fragment of Hsp90α or Hsp90β replaced (H9, H10, H13, and H14) grew at similar rate as the control strains that expressed native Hsp90α or Hsp90β, respectively (Fig. 2B). Therefore, we concluded that the fast/slow growth phenotype was not affected by the replacement of the charged linker region or the N-terminal fragment of the N-domains.

The remaining transformants could be divided into two groups clearly differing in their growth rates. Fast growing strains expressed Hsp90β or hybrids H3, H6, H7, whereas slow growing strains were transfected with Hsp90α or hybrids H4, H5, H8 (Fig. 2C). These results demonstrated that the fast growth of yeast cells correlated with the presence of the middle domain of the Hsp90 β in the analyzed hybrid gene. To narrow down this region, we analyzed hybrids in which only half of the middle domain was replaced (H11 and H12). The yeasts transformed with the hybrid H11, composed of Hsp90α with the C-terminal part of the middle domain replaced with the respective Hsp90β sequence, demonstrated fast growth phenotype, whereas the H12 hybrid composed of Hsp90β sequence and the C-terminal fragment of the Hsp90α middle domain conferred slow growth phenotype (Fig. 2C). In an attempt to identify residue(s) responsible for the slow growth phenotype of yeasts expressing H12, we constructed two additional hybrids (H15 and H16). Each of these hybrids contained only half of the Hsp90α sequence present in the H12 hybrid. Results presented in Fig. 2C demonstrate that growth of the yeasts that expressed H15 and H16 is comparable to the control strain that expresses wild-type Hsp90β. These results indicated that the yeast growth rate was

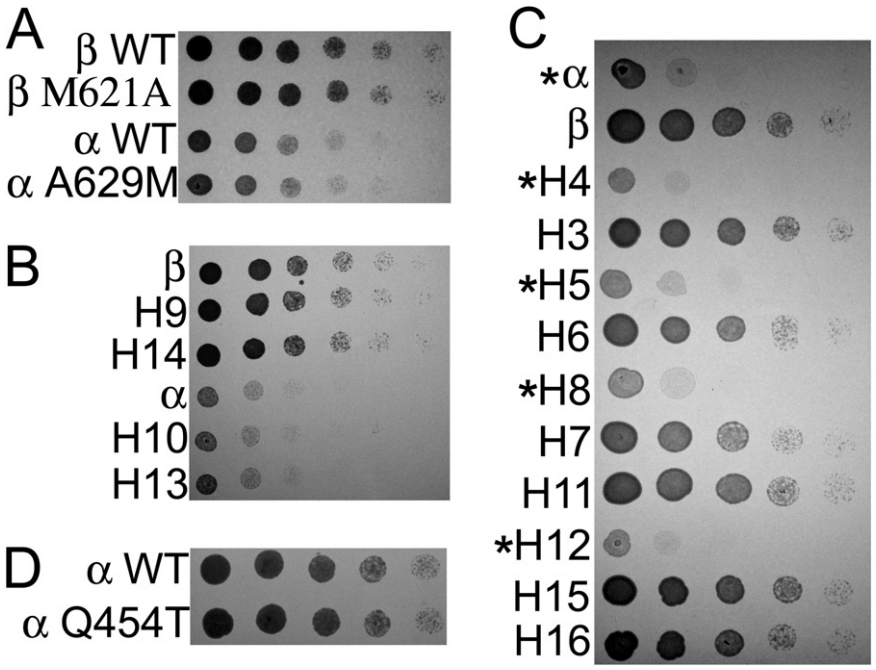

Fig. 2. Growth assay of yeasts that express Hsp90 hybrids and mutants. Saturated yeast cultures were diluted to equal optical density. Aliquots (2 μl) from the series of 2-fold dilutions (panels A, B and D) or 4-fold dilutions (panel C) of yeast cultures expressing indicated Hsp90 hybrids or Hsp90 mutants were spotted on YPD plates and incubated at 30 °C. Hybrids that contain amino acids 380–560 of Hsp90α on panel C are marked with an asterisk (*). Presented are results of one of three experiments.

determined by the overall structure of the c-terminal half of the human Hsp90 M-domain rather than by the single aa changes.

3.4. M-domain of Hsp90α binds Aha1 with high affinity

Published results suggest that Aha1 co-chaperone may bind to Hsp90α stronger than it does to Hsp90β [18,31]. This observation was confirmed by the results of the co-immunoprecipitation presented in Fig. 3a. Therefore, we attempted to attribute this difference to a specific region of the Hsp90 using hybrid Hsp90 genes. To this end, we constructed Hsp90 hybrids with a single aa substitution, namely, $Ile_{128}Thr$ in Hsp90α and the corresponding $Ile_{123}Thr$ in Hsp90β, which renders Hsp90 insensitive to compounds targeting ATP-binding pocket, such as geldanamycin or 17–AAG [31]. Hsp90 activity depends strictly on ATP hydrolysis. Cells transfected with the inhibitor-resistant mutant can be cultured in a presence of the 17-AAG. In such cells, endogenous Hsp90 should be inactive and the Hsp90 activity should come solely from the transfected Hsp90 protein. This inhibition of the endogenous Hsp90 allowed study of the transfected Hsp90 activity, without using standard gene-silencing techniques. To test whether the formation of heterodimers between native and transfected Hsp90 proteins could influence observed results, we compared expression ratio of the endogenous Hsp90β and FLAG-tagged Hsp90β $I_{123}T$ in the cells after 17-AAG selection and the composition of the complexes immunoprecipitated from these cells using anti-FLAG antibody. Similar to the previously published results [31], we found native and mutant proteins were expressed at the similar level, but the complexes isolated by the immunoprecipitation were composed predominantly of the tagged mutant protein Fig. 3b. Therefore, we concluded that heterodimerization of the native and mutant Hsp90 proteins should not pose substantial problem in the analysis of the mutant Hsp90.

HEK-293 cells transfected with different variants of the Hsp90 genes were selected using 17-AAG. The 17-AAG-resistant clones that expressed transfected Hsp90 were obtained in each case. This confirmed that all Hsp90 hybrids were functional. It has been shown previously that human Hsp90α/β hybrids with swapped N-domains are functional in yeast [38]. Our results proved that each domain of the human Hsp90 can be replaced with its homolog from the other isoform to create a functional protein. The 17-AAG toxicity was similar for all tested transfectants. IC50 values for all transfectants exceeded 2 μM, whereas control cells transfected with empty vector were unable to grow in 0.25 μM 17-AAG. We used 1 μM 17-AAG for the subsequent experiments because at this concentration all transfectants grew at a rate similar to the control cells grown in medium without 17-AAG and the cells maintained expression of the transfected Hsp90 even during prolonged culture (Supplementary Fig. 2).

Co-immunoprecipitation results indicated that differences in Aha1 binding to Hsp90 were determined by the M-domain of Hsp90 (Fig. 4A). Every Hsp90 protein that contained the Hsp90α M-domain bound more Aha1 than Hsp90 that contained the Hsp90β M-domain.

We also tested the binding of Cdc37, another Hsp90 co-chaperone, to hybrid Hsp90 proteins. Cdc37 bound to all the hybrids with no clear preference toward any particular isoform (Fig. 4A).

Results of our analysis of Hsp90 hybrids in yeasts suggested that C-terminal part of M-domain may discriminate between human Hsp90 isoforms. Therefore, we looked for aa differences in this region of Hsp90α and Hsp90β that could influence binding of Aha1 (Supplementary Fig. 1). The structure of the yeast Aha1 complex with the middle domain of Hsp90 suggests that only one of these residues, Glu_{454} in Hsp90α (corresponding to Thr_{446} in Hsp90β), is located at the Hsp90-Aha1 interface. This residue is homologous in the yeast Hsp82 sequence to Glu_{434} that interacts directly with Glu_{108} in the Aha1 [39]. However, results presented in Fig. 2D demonstrated that growth rates of the strain that expressed Hsp90α $Glu_{454}Thr$ mutant and of the control strain expressing the wild-type Hsp90α were similar.

3.5. Human Hsp90s require Aha1, but not Hch1, for the full activity in yeast cells

We hypothesized, based on the results of co-immunoprecipitation in human cells, that human Hsp90 isoforms may interact differently with the yeast homologues of human Aha1, named Aha1 and Hch1 [40,41]. To test this hypothesis, we tested the effect of the deletion of *AHA1* and *HCH1* genes on the growth of the strains BY561 and BY562 that expressed one of the human Hsp90 proteins. Results presented in Fig. 5 demonstrated that disruption of *HCH1* and *AHA1* had very little

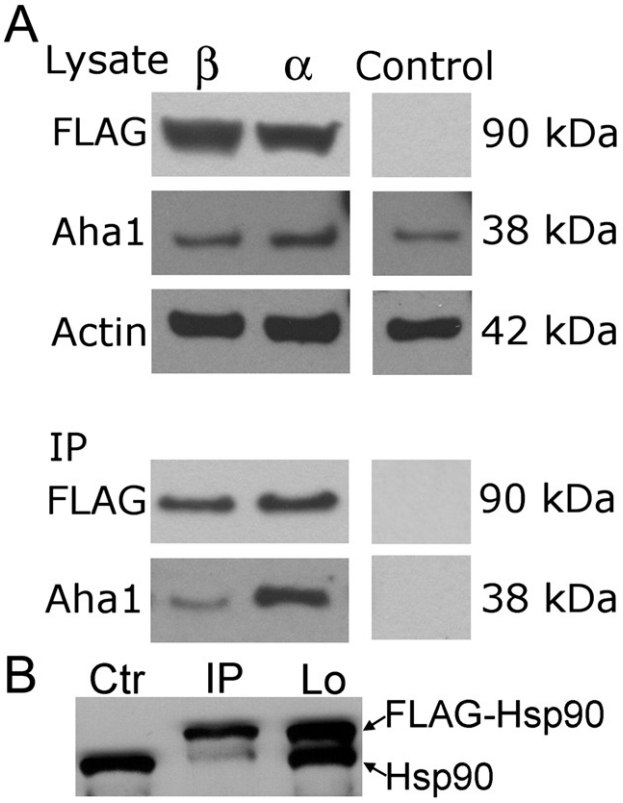

Fig. 3. Aha1 binds Hsp90α with higher affinity. (A) Hek 293 cells were transfected with plasmids that expressed wild-type Hsp90α and Hsp90β fused to N-terminal Flag tag. Immunoprecipitation was performed with Flag-specific antibody 48 h after transfection. Aha1 was detected in precipitates normalized for equal amount of Hsp90 protein. Control—immunoprecipitation made from the cells transfected with empty vector. (B) Hek 293 cells were transfected with plasmid that expressed Hsp90β $I_{123}T$. Transfectants were selected for resistance for 17-AAG. Immunoprecipitation with anti-FLAG antibody was performed using cells that were cultured in a presence of 1 μM 17-AAG. Lines are labeled as follows: Ctr—lysate from the HEK 293 cells not transfected and not treated with 17-AAG; IP—sample after immunoprecipitation; Lo—lysate used as a load for the immunoprecipitation. Western blot was detected with antibody specific for Hsp90β. Panels A and B presents results of one of two independent experiments.

effect on growth of the yeast expressing human Hsp90β. The growth of the strain that expressed Hsp90α was compromised by the disruption of the *AHA1* gene, but not by the disruption of the *HCH1* gene.

3.6. Hsp90 client protein cIAP1 interacts preferentially with the N-domain of Hsp90β

We reasoned that a substrate protein preferentially chaperoned by one Hsp90 isoform may interact differently with the Hsp90 hybrids allowing for identification of the Hsp90 region responsible for isoform specificity. We tested this hypothesis using as a model cIAP1 protein that is chaperoned by Hsp90β, but not Hsp90α [24]. Results presented in Fig. 4B demonstrate that cells transfected with the HSP90 gene that contained the N-domain of HSP90β express more cIAP1 than cells transfected with HSP90 that contained the N-domain of HSP90α.

4. Discussion

Most client proteins are believed to interact equally well with both human cytoplasmic Hsp90 isoforms, but some reports provide evidence for distinct roles for Hsp90α and Hsp90β in chaperoning of several proteins. Studies of specific functions of Hsp90 α and β isoforms in living cells using methods based on mRNA silencing are hindered by high expression and stability of these proteins. Therefore, even when mRNA for Hsp90 is eradicated, some residual level of the protein may remain. Another problem is posed by unequal expression level of Hsp90α and Hsp90β. Silencing of the prevalent isoform may have a stronger effect on the client protein simply because of the depletion of the total cellular Hsp90 pool rather than due to isoform-specific interactions. Despite this bias, some preferential interactions of client proteins with one Hsp90 isoform were confirmed [21,24,25,27,28,30]. However, at the molecular level, the structural bases for the observed specificity were not studied.

In this work, we exploited the differences in the capacity of human Hsp90 α and Hsp90β for supporting growth of yeast cells to identify structural features responsible for isoform specificity. Growth assays of the yeasts expressing hybrid Hsp90 genes composed of swapped Hsp90α and Hsp90β fragments demonstrated that the major difference between the Hsp90 isoforms is related to a small fragment of the middle domain. The slow growth phenotype associates with the presence of the aa 380-560 of Hsp90α in the hybrid. The corresponding fragment of the Hsp90β (aa residues 372-552) confers fast growth phenotype. It is worth to note that this fragment contains arginine residue crucial for the Hsp90 ATPase activity (Arg_{400} in Hsp90α). However, Arg_{400} is located in the loop that is strictly conserved in both human Hsp90 isoforms. Moreover, direct involvement of this structure in the isoform's differences is unlikely because Hsp90α and Hsp90β have similar ATP hydrolytic activity *in vitro* [42]. All non-conserved residues in the fragment sufficient for the change of the yeast growth rate are located in the 124 aa long fragment between aa 416 and 540 in Hsp90α (aa 408-532 in Hsp90β). The structure of M-domain is composed of two distinct domains with the αβα fold connected by three α-helical fragments [2]. The fragment of human Hsp90 identified in our yeast growth assays as sufficient and necessary to confer slow growth phenotype constitute smaller, C-terminal, αβα domain and α-coils connecting αβα domains. It is likely that the same structure is responsible for the isoform-specific differences in interaction with Aha1. Sequences of human Hsp90 are 80% identical (and 89% similar) in this region and differ in only 25 aa residues. Detailed structural data for the human Hsp90 proteins are not available. It is therefore difficult to speculate why these subtle differences significantly impact Hsp90 activity.

Hsp90 undergoes extensive post-translational modifications, such as phosphorylation, acetylation, S-nitrosylation, and ubiquitination [43]. Some of them were shown to modify Hsp90–Aha1 interaction. Phosphorylation of Hsp90α on Tyr_{38} and SUMOylation of Lys_{191} stimulates Aha1 binding, whereas acetylation of Lys_{294} on Hsp90α decreases Aha1 binding [35,44,45]. The influence of these modifications on Hsp90β activity was not studied. The modified residues are conserved in human Hsp90β (Tyr_{33}, Lys_{186}, and Lys_{286}) and in both yeast Hsp90 (Tyr_{24}, Lys_{178}, and Lys_{270}), and the effects of the modifications at these residues are similar in both species, which makes them an unobvious target of isoform-specific post-translational modifications. However, the possibility of the existence of regulatory mechanisms that result in differential modifications of these residues cannot be excluded. Recently, it was demonstrated that expression of constitutively active met and v-src leads to increased SUMOylation of Hsp90 [45]. One might speculate, by analogy to this observation, that isoform-specific regulation of the post-translational modifications is based on Hsp90 interaction with co-chaperones, or on Hsp90 preference for client proteins.

The only known post-translational modification restricted to one human isoform is phosphorylation of Hsp90α on Thr5 and Thr7 because these residues have no homologues in the Hsp90β [46,47]. Our experiments excluded the possibility that these modifications are responsible for the growth differences in yeast. The sequence comparison of Hsp90α aa fragment 416–540 and the corresponding fragment of Hsp90β suggest several residues that are not conserved in other isoform and that could be a subject of an isoform-specific post-translational modification. The most likely candidates are Lys_{457}, Lys_{456}, Lys_{478}, and Thr_{466} in Hsp90α and Thr_{446}, Thr_{467}, Thr_{479}, Ser_{470}, and Ser_{482} in Hsp90β. Whether modifications of any of these sites in human cells

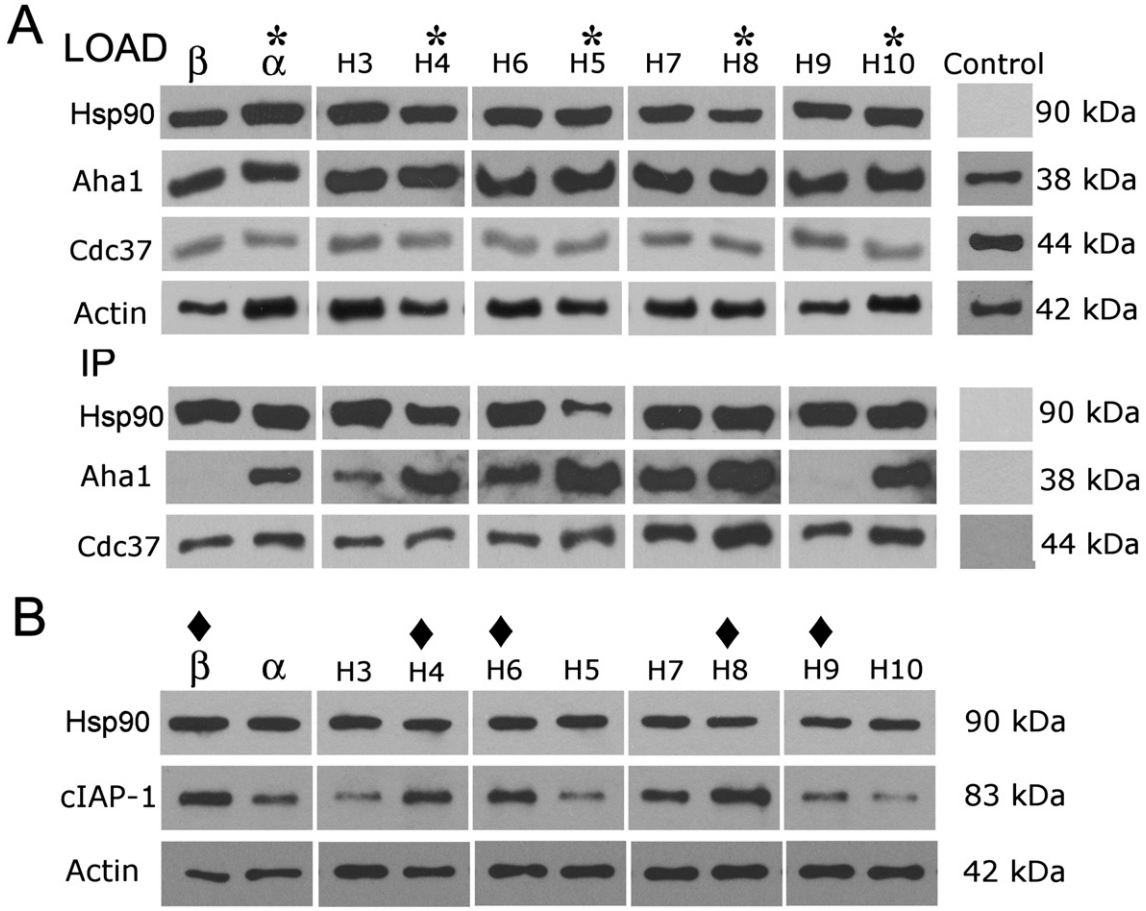

Fig. 4. Aha1 binds preferentially to Hsp90α M-domain, and cIAP1 is chaperoned more efficiently by Hsp90β N-domain. (A) Hek 293 cells transfected with the indicated Hsp90 alleles that carried mutation I123T or I128T and the N-terminal FLAG peptide were selected for 17-AAG resistance. Lysates made of cells that stably express indicated Hsp90s were immunoprecipitated with anti-Flag Ab. Control represents IP made from the Hek 293 cells transfected with plasmid pcDNA3.1 and selected for resistance to G418. Aha1 and Cdc37 bound to Hsp90 was detected using specific antibodies. Presence of the α M-domain in the transfected Hsp90 gene is marked with an asterisk (*). (B) Cells transfected and selected as in panel A were transfected with second plasmid that carried cIAP1 gene fused to the c-terminal myc-tag. CIAP1 expression was detected in lysates 48 h after transfection. Hybrids that contain Hsp90β N-domain are marked with ♦. Figures show results of one of two independent experiments.

are involved in structural differences in the C-terminal region of the M-domain that change its affinity for Aha1 remains an open question. However, to explain the different genetic interaction of Aha1 with human Hsp90 isoforms in yeast by post-translational modifications it is necessary to assume that yeasts are capable of the isoform-specific modifications of the human Hsp90 the same way human cells are. Moreover, this mechanism would have to evolve independently in both species (or their ancestors) because the last common ancestor of yeast and humans had only one Hsp90 gene, and pairs of Hsp90 genes in humans (Hsp90α and Hsp90β) and *S. cerevisiae* (Hsc82 and Hsp82) evolved as a result of independent duplications [48]. Such parallel evolution is unlikely. Therefore, Hsp90 isoform-specific effects in yeast are more easily explained by differences in the aa composition and structural differences of Hsp90α and Hsp90β M-domain.

Aha1 stimulates ATP hydrolysis by the N-domain of Hsp90 and activates the chaperoning activity of Hsp90 [49]. It was previously shown that both the M- and N-domains of Hsp90 contribute to its interaction with Aha1 [41,50]. Our results demonstrate that in human cells Aha1 binds stronger with the Hsp90 hybrids that contained the M-domain of Hsp90α. It was shown previously that K_d for ATPase stimulation by Aha1 is 3.5-fold lower for Hsp90α compared to K_d for Hsp90β, but V_{max} for Hsp90β is 2-fold higher than V_{max} for Hsp90α [42]. The higher V_{max} for Hsp90β most likely led to the underestimation of K_d value for this isoform because Aha1 binding to Hsp90 was measured indirectly by ATPase hydrolysis. Therefore, the real value of K_d for Aha1 binding to Hsp90α may be 7-fold lower than K_d for Hsp90β. The response of Hsp90α and Hsp90β to changes in Aha1 concentration may be influenced by their differing affinity for Aha1. This regulatory mechanism may be important when Aha1 expression changes under stress conditions or as a result of regulation by other proteins [49,51].

We used Hsp90 alleles with N-domain mutations that increase the resistance of Hsp90 to inhibitors such as 17-AAG in experiments in HEK-293 cells. By culturing the transfected cells in medium containing 17-AAG, we were able to select population of the cells that expressed transfected Hsp90 at very high level, matching that of the endogenous Hsp90 [31]. Moreover, the inhibitor inactivated dimers composed of the endogenous Hsp90, leaving protein expressed from the transfected gene as the only functional Hsp90 in the cell. However, heterodimers composed of the native and transfected Hsp90 could form even in the presence of the inhibitor. We did observe such heterodimers, but they constituted a minor fraction of the immunoprecipitated complexes. Most of the Hsp90 dimers were composed of two subunits of the tagged Hsp90 expressed from the transfected plasmid. Hsp90 dimerization is driven primarily by the C-domains. However, changes in ability of the N-domains to bind ATP and to adopt closed conformation affect stability of Hsp90 dimers [52]. Therefore, an inhibitor bound to the ATP-binding site of the native Hsp90 could destabilize dimer. That might explain preferential formation of the dimers composed of the two molecules of the inhibitor-resistant Hsp90.

The C-domain of the hybrid Hsp90 determines which isoform of the native Hsp90 may form heterodimer with a given hybrid Hsp90.

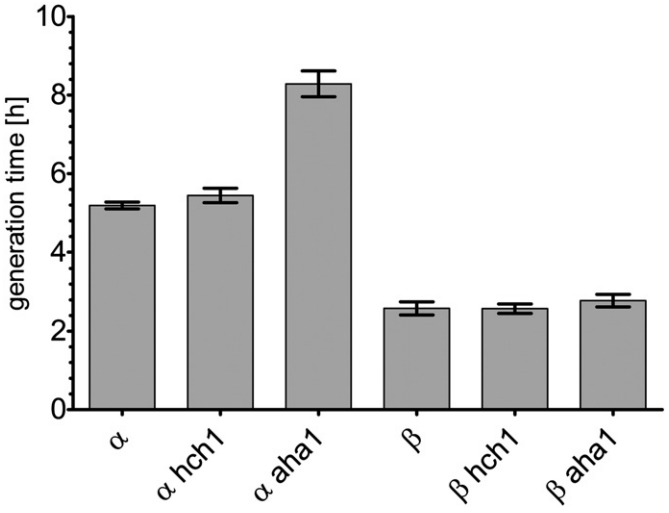

Fig. 5. Hsp90α activity in yeast depends on Aha1. *AHA1* and *HCH1* genes were disrupted in the yeast strains that expressed Hsp90α or Hsp90β. The growth of these strains was measured in YPD medium at 30 °C using Bioscreen C microplate reader (Oy Growth Curves Ab Ltd). Plot presents generation times and SD calculated from four experiments.

Hsp90α Ile_{128}Thr and hybrids containing the C-domain of hsp90α ($H4^{IT}$, $H6^{IT}$, $H7^{IT}$, and $H10^{IT}$) may form heterodimers with native Hsp90α. Similarly, Hsp90β Ile_{123}Thr and hybrids $H3^{IT}$, $H5^{IT}$, $H8^{IT}$, and $H9^{IT}$ may form heterodimers with native Hsp90β. The resulting bias should be similar for all hybrids that dimerize with the same native Hsp90 isoform and should decrease observed differences in Aha1 binding rather than enhance them. Minor influences of the native Hsp90 cannot be excluded in some of the results. For example, low binding of Aha1 to H8, compared to H7, may be caused by heterodimerization with native Hsp90β and rather high binding of Aha1 to H6, compared to H5, could be explained by formation of heterodimers with native Hsp90α. However, even in these cases the effect of M-domain clearly prevails because binding of Aha1 to the hybrids with the α M-domain is stronger.

The Hsp90α Ile_{128}Thr and Hsp90β Ile_{123}Thr aa substitutions, located in the N-domains of these proteins, greatly increase affinity of the mutant Hsp90 to Aha1, which could raise concerns of possible artifacts caused by these mutations [31]. However, these mutations increase Aha1 binding to both Hsp90 isoforms [53]. Therefore, difference in affinity for Aha1 demonstrated by the Hsp90α and Hsp90β M-domains is unlikely to result from these single aa substitutions. Two distinct observations validate the results obtained with Hsp90 17-AAG-resistant mutants. Namely, (i) Cdc37, which is a co-chaperone that interacts with the N-domain, bound to both Hsp90α Ile_{128}Thr and Hsp90β Ile_{123}Thr with equal affinity [54]. (ii) Non-mutated Hsp90α bound *in vivo* Aha1 with higher affinity when compared to binding with Hsp90β.

There are two homologues of human *AHA1* in the yeast genome, called *AHA1* and *HCH1*. The longer protein encoded by *AHA1* corresponds to the full-length human protein, whereas *HCH1* encodes a shorter protein that corresponds to the N-domain of the human Aha1. Yeast Aha1 is only 23% identical to its human homolog and Hch1 is even less conserved [41]. Deletion of either gene has no effect on yeast grown at 30 °C and becomes detrimental only when cells are exposed to elevated temperatures and are forced to respiratory growth [49]. These results suggest that yeast Hsp90 proteins can function effectively without aid from these co-chaperones and the cooperation between Hsp90 and Aha1 or Hch1 becomes necessary only under stress caused by high temperature or growth conditions. Our experiments demonstrated that deletion of *AHA1* or *HCH1* genes had little effect on yeasts expressing human Hsp90β. This isoform, like its yeast homolog, does not depend on the ATPase stimulating co-chaperones under non-stressful conditions whereas Hsp90α under the same conditions requires Aha1 for full activity. In the yeast cell for each Aha1 or Hch1 molecule, there are at least 12 Hsp90 dimers (counting together Hsc82 and Hsp82) [55]. If the expression rates of Aha1 and Hsp90 in human cells are similar, Hsp90 must compete for the available co-chaperone. The experiments in yeast suggest that chaperoning activity of Hsp90α is more dependent on Aha1 than activity of Hsp90β. Therefore, the high affinity of Hsp90α for Aha1 observed in human cells may be an adaptation that allows this isoform to function effectively in a presence of limiting quantities of the co-chaperone. Hch1 has no human homolog, is only 36% identical to the yeast Aha1, and is a weak stimulator of the yeast Hsp90 ATPase compared to Aha1. Moreover, despite their homology, Aha1 and Hch1 seem to interact with Hsp90 in a different way [56]. Therefore, Hch1 may not be able to interact with human Hsp90s or may not stimulate their ATPase activity. That may explain why *HCH1* deletion does not affect growth of yeast that express human Hsp90 as a sole source of this protein.

CIAP1 is the specific substrate for Hsp90β [24]. An interaction with Hsp90β stabilizes cIAP1 and increases its cellular concentration. Client proteins are believed to interact with the middle domain of Hsp90 [2,57]. Therefore, we expected that cIAP1 would be chaperoned more efficiently by the hybrids that contain the middle domain of this isoform. Surprisingly, the high expression level of cIAP1 depended on the presence of N-terminal but not M-domain of Hsp90β in the hybrid gene.

Structural analysis of the Hsp90-Cdc37-Cdk4 complex suggested that the N-domain of Hsp90 contributes to the binding of the client kinase Cdk4 [58]. Similarly, cIAP1 may also interact directly with both N and M-domain of Hsp90, and the preference of cIAP1 for Hsp90β may result from some small differences in structure of the highly homologous α and β N-domains.

In conclusion, we demonstrated for the first time that Aha1 co-chaperone interacts preferentially with Hsp90α and that this specific interaction is mediated by the M-domain of Hsp90. We also provided evidence for the specific interaction of cIAP1 with the N-domain of Hsp90β. Aha1 and cIAP1 use different fragments of Hsp90 to discriminate between Hsp90α and Hsp90β, suggesting that functional differences between Hsp90 isoforms may depend on differences dispersed among many fragments of this protein. Specificity of the interaction with a particular protein may depend on a set of structural features unique for this protein. Our results proved also that using 17-AAG-resistant mutants as an alternative to the conventional techniques used for protein expression silencing facilitates functional Hsp90 analysis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.11.026.

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