Substitution of only two residues of human Hsp90 α causes impeded dimerization of Hsp90 β

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Abstract Two isoforms of the 90-kDa heat-shock protein (Hsp90), i.e., Hsp90 α and Hsp90 β , are expressed in the cytosol of mammalian cells. Although Hsp90 predominantly exists as a dimer, the dimer-forming potential of the β isoform of human and mouse Hsp90 is less than that of the α isoform. The 16 amino acid substitutions located in the 561–685 amino acid region of the C-terminal dimerization domain should be responsible for this impeded dimerization of Hsp90 β (Nemoto T, Ohara-Nemoto Y, Ota M, Takagi T, Yokoyama K. *Eur J Biochem* 233: 1–8, 1995). The present study was performed to define the amino acid substitutions that cause the impeded dimerization of Hsp90 β . Bacterial two-hybrid analysis revealed that among the 16 amino acids, the conversion from Ala₅₅₈ of Hsp90 β to Thr₅₆₆ of Hsp90 α and that from Met₆₂₁ of Hsp90 β to Ala₆₂₉ of Hsp90 α most efficiently reversed the dimeric interaction, and that the inverse changes from those of Hsp90 α to Hsp90 β primarily explained the impeded dimerization of Hsp90 β . We conclude that taken together, the conversion of

Thr₅₆₆ and Ala₆₂₉ of Hsp90 α to Ala₅₅₈ and Met₆₂₁ is primarily responsible for impeded dimerization of Hsp90 β .

Keywords Hsp90 · Dimerization · Amino acid substitution · Molecular chaperone

Abbreviations

Hsp	heat-shock protein
Hsp90	90-kDa heat shock protein
hHsp90	human Hsp90
hHsp90 α and hHsp90 β	α and β isoforms, respectively, of hHsp90
Grp94	94-kDa glucose-regulated protein/endoplasmic reticulum paralog of Hsp90
Trap1	Trap1/mitochondrial paralog of Hsp90
HtpG	bacterial ortholog of eukaryotic Hsp90
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate

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Introduction

The 90-kDa heat shock protein (Hsp90) molecular chaperone is either transiently or stably associated with specific client proteins that are unstable unless chaperoned by Hsp90. Prokaryotes express a single Hsp90-family protein, designated to HtpG (Bardwell and Craig 1987); whereas eukaryotic members of the Hsp90-family of proteins are widely distributed to subcellular compartments. The 94-kDa glucose-regulated protein (Grp94) is expressed in the endoplasmic reticulum (Pelham 1986), Trap1 exists in

mitochondria (Song et al 1996; Felts et al 2000), and the family protein is also located in the plastids of plant cells (Schmitz et al. 1996). In yeast cytosol, two Hsp90 isoforms, designated as Hsp82 and Hsc82, are expressed (Ferrelly and Finkelstein 1984; Borkovich et al 1989). In mammalian cells, two isoforms of the cytosolic Hsp90, called Hsp90 α (hHsp90 α) and Hsp90 β (hHsp90 β) in humans (Rebbe et al 1987; Hickey et al 1989) and Hsp86 and Hsp84 in mice (Moore et al 1989), are expressed. Mouse Hsp86 and Hsp84 are orthologs of hHsp90 α and hHsp90 β , respectively. The amino acid sequence of hHsp90 α is 85.5% identical to that of hHsp90 β . To date, the functional difference between the two Hsp90 isoforms is not known. Both of them are associated with the glucocorticoid receptor (Mendel and Orti 1988). Either one of the isoforms is required for the growth of yeast at elevated temperatures (Borkovich et al. 1989).

Hsp90 is a dimeric protein (Welch and Feramisco 1982). According to the functional model of the Hsp90 molecular chaperone, the C-terminal dimeric region functions as a hinge for a molecular clamp, in which a pair of the client-binding sites are located within the N-terminal domain and/or middle domain of Hsp90 (Prodromou et al. 1997; Meyer et al. 2003). The C-terminal 191 amino acids of hHsp90 α (amino acids 542–732) are sufficient for the dimerization (Nemoto et al. 1995); and amino acids 662–678, having hydrophobicity, are indispensable for this role (Yamada et al. 2003).

Only a single physicochemical difference is known as regards mammalian Hsp90 isoforms. Both mouse and human Hsp90 β possess an impeded dimerization activity compared with the dimerization activity of Hsp90 α (Minami et al. 1991; Minami et al. 1994; Nemoto et al. 1995). Among the C-terminal 191 amino acids of hHsp90 α sufficient for the dimerization, the extreme C-terminal end at residues 698–732, which contains 12 amino acid differences between Hsp90 isoforms, was unrelated to the impeded dimer formation of hHsp90 β (Nemoto et al. 1995). Accordingly, the residual 16 differences located at residues 542–685 appeared to be responsible for this phenomenon.

In the present study, we determined the amino acid substitutions responsible for the impeded dimer formation of hHsp90 β . The results of bacterial two-hybrid analysis revealed two amino acid substitutions primarily responsible for this phenomenon.

Materials and methods

Materials

The materials used and their sources were as follow: expression vector pQE9 and plasmid pREP4, from Qiagen Inc. (Chatsworth, CA, USA); expression vector pTrcHis

TOPO and TOP10 competent cells, from Invitrogen (Carlsbad, CA, USA); and Talon metal affinity resin, from Clontech Laboratories Inc. (Palo Alto, CA, USA).

Nomenclature and amino acid number

According to our previous results on limited proteolysis, we divided hHsp90 α into three domains (Nemoto et al. 1997): Amino acid residues 1–400, 401–618, and 619–732 correspond to the N, M, and C domains, respectively. The domain border between the N and M domains is distinct from that defined by Stebbins et al. (1997) or by Prodromou et al. (1997). Our definition of the N domain is based on the cleavage site most susceptible to limited proteolysis, whereas the others defined the N-terminal core region (residues 1–220/230) as that which is highly resistant to proteolysis. In the present study, truncated forms of the two Hsp90 species used for the two-hybrid analysis were expressed based on domain units, which were referred to as M, C, and MC for the M domain, C domain, and MC domain, respectively.

hHsp90 α and hHsp90 β are composed of 732 and 724 amino acids, respectively. The difference is caused by the deletion of amino acids in hHsp90 β corresponding to residues 9–13 and 241–243 of hHsp90 α . Accordingly, Thr₅₆₆ and Ala₆₂₉ of hHsp90 α , respectively, are equivalent to Ala₅₅₈ and Met₆₂₁ of hHsp90 β .

Construction of the full-length forms of the Hsp90-family members

Construction of plasmids encoding the full-length forms of hHsp90 α (Nemoto et al. 1996), hHsp90 β , human Grp94 (Roi 1998), HtpG from *Escherichia coli* (Nemoto et al. 2001) tagged with a histidine hexamer constructed with pQE9 (Qiagen Inc., Chatsworth, CA, USA) was described previously. Y1090[pREP4] was transformed with the plasmids, and the transformants were selected on Luria-Bertani agar plates containing 50 μ g/ml of ampicillin and 25 μ g/ml of kanamycin. Construction of plasmids encoding the full-length forms of HtpG from *P. gingivalis* and human Trap1 constructed with pTrcHis TOPO (Invitrogen) was described previously (Kawano et al. 2004). The full-length form of hHsp90 β was amplified by polymerase chain reaction (PCR) with sense primer 5'-CTTGGATCC ATGCCTGAGGAAGTGCACCA and antisense primer 5'-ACTGTGCGACCTAATCGACTTCTTC and directly inserted in pTrcHis TOPO according to the manufacturer's recommendation. TOP10 cells transformed with the plasmids were selected on Luria-Bertani agar plates containing 50 μ g/ml of ampicillin. The insertion and orientation of the DNA fragments were confirmed by conducting Hot Star PCR (Qiagen Inc., Chatsworth, CA, USA).

Expression and purification of recombinant proteins

After overnight cultivation of the transformed bacteria at 37°C, recombinant proteins were expressed at 30°C for 4 h in the presence of 0.2 mM isopropyl- β -D-thiogalactopyranoside. They were purified by affinity chromatography on a Talon affinity column according to the manufacturer's protocol except that 10 mM imidazole was included in the lysis/washing buffer. After extensive washing to remove non-adsorbed substances, bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol.

Bacterial two-hybrid system

The dimerization activity was quantified by the bacterial two-hybrid system according to Karimova et al. 2001. Construction of pKT25-hHsp90 α carrying the M domain [pKT25-hHsp90 α -M (amino acids 401–618)], the MC domain [pKT25-hHsp90 α -MC (amino acids 401–732)], and the C domain [pKT25-hHsp90 α -C (amino acids 619–732)] and pUT18C-hHsp90 α carrying MC [pUT18C-hHsp90 α -MC] was described previously (Yamada et al. 2003; Kawano et al. 2004). For construction of pKT25-hHsp90 β -MC and -C, the corresponding regions were amplified by PCR with appropriate primers, and then inserted in an *XbaI*-*SmaI* site of pKT25. For construction of pKT25-hHsp90 β -M, the DNA fragment encoding the M domain was amplified by PCR with appropriate primers, and then, inserted into a *PstI*-*BamHI* site of pKT25. *E. coli* strain BTH101 was co-transformed with pKT25- and pUT18C-derived plasmids. Complex formation between co-expressed proteins was distinguished by color development of the colonies on the MacConkey indicator media agar plates containing 1% (w/v) maltose. Quantitative data were obtained by the measurement of the β -galactosidase activity of their liquid cultures as described previously (Tanaka et al. 2001). Values were expressed as mean + SD. Statistical significance was evaluated by Student's *t* test for two samples.

In vitro mutagenesis

Sixteen amino acids distributed to residues 561–685 encoded by pKT25-hHsp90 β -MC were exchanged to the corresponding amino acids in the Hsp90 α sequence by PCR-based site-directed mutagenesis in combination with *DpnI* degradation of the template DNA (see Fig. 2). Inversely, Thr₅₆₆ encoded by pKT25-hHsp90 α -M, which was equivalent to Ala₅₅₈ of hHsp90 β , was substituted to Ala and Ala₆₂₉ encoded by pKT25-hHsp90 α -C, which was equivalent to Met₆₂₁ of hHsp90 β , and was substituted to Met as described above.

Random mutation at Ala₅₅₈ or Met₆₂₁ was introduced into pKT25-hHsp90 β -MC by PCR according to the method of Imai et al. (1991). For the mutagenesis of nucleotides encoding Ala₅₅₈, 5'-NNNAAGTTTGAGAACCTCTGCAAGC-3' (N=A, G, T, or C) and 5'-CTTGCTCTCTTCCATCTTCTTCTTC-3', we used the respective 5'- and 3'-primers. For THE mutagenesis of nucleotides encoding Met₆₂₁, 5'-NNNGCCAAAAAGCACCTGGAGATCA-3' and 5'-CATATAGCCCATGGTGGA GTTGT-3' were used as 5' and 3' primers, respectively. Following PCR with pKT25-hHsp90 β -MC as a template, the PCR fragments were blunt-ended, phosphorylated, and then ligated. BTH101[pUT18-hHsp90 α -MC] competent cells were transformed with the DNA. Transformed cells were grown up on MacConkey agar plates containing 1% (w/v) maltose. After a culture period of 3–5 days, red colonies were cloned for DNA sequencing and quantification of the β -galactosidase activity as described above.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in the presence of 0.1% (w/v) SDS at a polyacrylamide concentration of 12.5% (w/v). Proteins were stained with Coomassie brilliant blue. Low molecular weight markers (GE Healthcare UK Ltd.) were used as molecular markers.

To estimate the molecular configuration of proteins, we subjected samples to PAGE under nondenaturing conditions (Nemoto et al. 1995). Electrophoresis was performed at low voltage (50 V) at room temperature to avoid elevation of the gel temperature. Proteins were separated on a 7.5% (w/v) polyacrylamide gel and then stained with Coomassie brilliant blue. Ovalbumin (45 kDa), bovine serum albumin (66 kDa as monomer; 132 kDa as dimer, and 198 kDa as trimer), and catalase (240 kDa) were used as molecular references.

Protein concentration

Protein concentrations were determined by use of the bicinchoninic acid method using bovine serum albumin as a standard (Pierce, Rockford, IL, USA).

Results

Comparison of the dimer formation by Hsp90-family members

The dimer-forming potentials of mouse and human Hsp90 β are lower than those of their respective Hsp90 α . However, it is not known whether the impeded dimerization is specific to mammalian Hsp90 β . Hence, we compared the

dimer-forming activity of the recombinant form of the Hsp90-family members from various species. They were expressed by pQE (Qiagen) and pTrcHis TOPO vectors (Invitrogen), which carried the 12- and 35-amino acid peptides, respectively, at the N-terminus.

Sodium dodecylsulfate-PAGE revealed that hHsp90 α (Fig. 1a, lane 1), HtpG from *Escherichia coli* (lane 4), human Trap1 (lane 6) and HtpG from *Porphyromonas gingivalis* (lane 7) were purified to near homogeneity and that hHsp90 β (lanes 2 and 5) and human Grp94 (lane 3) contained additional bands corresponding to molecular weights smaller than the molecular weight for the major species. As reported previously (Roi 1998), the preparation of human Grp94 (lane 3) contained a 53-kDa species, which was a C-terminal portion of hGrp94 starting at Met336. The N-terminal sequences of small molecular-mass species found in the preparation of hHsp90 β (35 kDa in lane 2 and 38 kDa in lane 5) failed to be determined. Because hHsp90 β was expressed with the two expression vectors, the difference in the mobility between the two species (compare lanes 2 and 5) appears to reflect the length of the N-terminal tag peptides. Thus, it is reasonable to suggest that the smaller species were the C-terminally truncated hHsp90 β .

To examine their configurations, we separated the purified proteins on PAGE under non-denaturing conditions. As reported previously (Nemoto et al. 1995), hHsp90 α migrated as a dimer (Fig. 1b, lane 1). Similarly, human Grp94, human Trap1, and HtpG from *P. gingivalis* predominantly migrated as dimers. HtpG from *E. coli* also migrated as a dimer; although it migrated much faster than the dimers of other members, thus reflecting its lower molecular mass ($M_r=71,429$: 624 amino acids). In contrast, hHsp90 β was split into two bands (lane 2). The upper

minor one corresponded to the dimer; and the lower major one to the monomer. This electrophoretic pattern did not vary when hHsp90 β was expressed with the second vector (lane 5). A band at the position equivalent to that of the hHsp90 β monomer was also observed for human Grp94 (lane 3), but the ratio of its density to that of the dimer was much less than in the case of hHsp90 β . These findings demonstrate that hHsp90 β had the least potent dimerization activity among the family members tested in Fig. 1. In some preparations of hHsp90 β , an additional band migrating faster than ovalbumin (a marker protein) appeared (lane 5). This band would seem to correspond to the 38-kDa form of hHsp90 β with a C-terminal truncation.

Evaluation of the dimeric interaction by use of the two-hybrid system

We compared the dimer-forming potential of hHsp90 isoforms by use of the bacterial two-hybrid system. Because we previously reported that the C-terminal 191 amino acids are sufficient for the dimer formation and that the region represents the impeded dimer forming ability of Hsp90 β (Nemoto et al. 1995), we decided to compare the activity of MC domain. Moreover, to narrow the region responsible for the difference in the dimer-forming activity, we quantified the binding potentials of the M and C domains to MC domain separately. Hsp90 α -MC was used as a binding partner, because the bindings to Hsp90 β -MC were much lower than those to Hsp90 α -MC (data not shown). As shown in Table 1, hHsp90 α -C efficiently bound to hHsp90 α -MC and hHsp90 β -C bound to it less efficiently. Moreover, the binding of hHsp90 β -M to hHsp90 α -MC was poor compared with that of hHsp90 α -M. Therefore, the impeded dimerization was faithfully reproduced by the two-hybrid system. Table 1 also demonstrates that the impeded dimer-forming activity of hHsp90 β was accomplished by at least two sites, one within the M domain and the other in the C domain.

Comparison of amino acids substituted in the dimer-forming region

At the region of the C-terminal 191 amino acids, there are 28 amino acid substitutions between hHsp90 α and hHsp90 β (Fig. 2). The 12 amino acid substitutions clustered near the C-terminus (residues 702–725) are known not to be responsible for the impeded dimerization of hHsp90 β (Fig. 2, plus signs). Thus, the remaining 16 substitutions occurring in the span of residues 561–685 should be responsible for this phenomenon (Fig. 2, asterisk). As the border between the M and C domains is Ala₆₁₈–Leu₆₁₉, hence the first nine amino acids belong to the M domain, and the remaining 7 to the C domain.

Fig. 1 Polyacrylamide gel electrophoresis of the Hsp90-family members – Recombinant forms of six Hsp90-family members were electrophoresed by SDS-PAGE (a) and by PAGE under non-denaturing conditions (b). Lane 1, hHsp90 α ; lane 2, hHsp90 β ; lane 3, human Grp94; lane 4, HtpG from *E. coli*; lane 5, hHsp90 β ; lane 6, human Trap1; lane 7, HtpG from *P. gingivalis*; and lane M, molecular marker markers. Purified proteins (0.5 and 1 μ g proteins, respectively) were loaded onto the gel in panels a and b

Table 1 Two-hybrid analysis of dimeric interaction of hHsp90 isoforms

pKT25-	pUT18C-	Activity (%) ^a
pKT25	pUT18C	14.2±4.2
hHsp90α-C	hHsp90α-MC	100.0±4.2
hHsp90β-C	hHsp90α-MC	29.1±12.1*
hHsp90α-M	hHsp90α-MC	105.2±0.6
hHsp90β-M	hHsp90α-MC	13.5±1.7*

^a Mean + SD (*n*=4)**p*<0.001. *P* values were calculated in comparison with respective controls (hHsp90α).

When 16 amino acids of MC domain of hHsp90β were substituted to those of hHsp90α, in two cases (Ala₅₅₈ and Met₆₂₁ of hHsp90β), there were significant increases in the interaction comparable to that of hHsp90α-MC (Table 2). Moderate enhancement was observed in three cases (Met₅₅₃, Ser₅₈₆, and Val₆₅₆ of hHsp90β). In remaining 11 cases, there were little changes. Thus, the binding was completely recovered by a single substitution at position 558 or 621 of hHsp90β.

Effect of reverse conversion of Thr₅₆₆ and Ala₆₂₉ of hHsp90α to Ala₅₅₈ and Met₆₂₁ of hHsp90β

Table 2 demonstrated that amino acid substitutions of hHsp90β at positions 558 and 621, which are located within M and C domains, respectively, appeared to be most significantly relevant to the impeded dimerization. In fact, Table 1 indicated that amino acid substitutions at both M and C domains were responsible for the impeded dimerization of hHsp90β. To evaluate the significance of the amino acid substitution at the two positions, we further

Fig. 2 Comparison of the amino acid sequences between hHsp90α and hHsp90β – The C-terminal 191 amino acids of hHsp90α and hHsp90β are compared. Numbers are represented as those of hHsp90α. Amino acid substitutions that are potentially involved in the impeded dimerization of hHsp90β are indicated by asterisks and 12 substitutions at the C-terminus that are not involved in the impeded dimerization are represented by pluses (Nemoto et al 1995). The border between M and C domains is Ala₆₁₈–Leu₆₁₉

Table 2 Effect of the amino acid conversion from hHsp90β to hHsp90α on the dimeric interaction

pKT25	Substitution		pUT18C	Activity (%) ^{a..}
	From	To		
pKT25			pUT18C	7.8±0.2
hHsp90α-MC			hHsp90α-MC	100.0±7.1***
hHsp90β-MC			hHsp90α-MC	30.3±13.1 ^b
1 hHsp90β-MC	Met ₅₅₃	Gln ₅₆₁	hHsp90α-MC	77.8±37.4*
2	Ser ₅₅₆	Lys ₅₆₄	hHsp90α-MC	32.2±16.0
3	Ala ₅₅₈	Thr ₅₆₆	hHsp90α-MC	109.9±20.4***
4	Leu ₅₆₆	Ile ₅₇₄	hHsp90α-MC	35.8±10.0
5	Glu ₅₆₉	Asp ₅₇₇	hHsp90α-MC	31.8±9.6
6	Asp ₅₇₂	Glu ₅₈₀	hHsp90α-MC	41.1±8.0
7	Thr ₅₇₉	Val ₅₈₇	hHsp90α-MC	24.5±9.3
8	Ile ₅₈₀	Val ₅₈₈	hHsp90α-MC	15.8±4.4
9	Ser ₅₈₆	Thr ₅₉₄	hHsp90α-MC	69.5±12.2**
10	Met ₆₂₁	Ala ₆₂₉	hHsp90α-MC	111.9±24.2***
11	Pro ₆₃₃	Ser ₆₄₁	hHsp90α-MC	28.6±6.6
12	Val ₆₃₅	Ile ₆₄₃	hHsp90α-MC	35.0±8.2
13	Ala ₆₅₀	Ser ₆₅₈	hHsp90α-MC	39.5±9.8
14	Val ₆₅₆	Ile ₆₆₄	hHsp90α-MC	67.5±17.6*
15	Phe ₆₅₉	Tyr ₆₆₇	hHsp90α-MC	38.3±11.2
16	Ser ₆₇₇	Ala ₆₈₅	hHsp90α-MC	35.1±8.5

Sixteen amino acids of hHsp90β-MC were substituted to corresponding amino acids of hHsp90α. Because of the deletion in hHsp90β corresponding to amino acids 9–13 and 241–243 of hHsp90α, Met₅₅₃ of hHsp90β is equivalent to Gln₅₆₁ of hHsp90α and so on.

^a Mean + SD (*n*=5)^b *P* values were calculated in comparison with the value of hHsp90β-MC. **p*<0.05, ***p*<0.01, ****p*<0.001

evaluated the effect of the changes on the dimer formation. If the substitution of the two amino acids is truly critical, the reverse conversion of the amino acids from those of hHsp90α to hHsp90β would be expected to reduce the binding. In fact, the binding activity of hHsp90α-MC Ala₅₆₆ to hHsp90α-MC was decreased to that which is comparable to the binding activity of hHsp90β-M (Table 3). The activity of hHsp90α-C Met₆₂₉ also decreased to some extent (Table 3). Therefore, we conclude that the substitutions of these two amino acids are primarily, if not completely, responsible for the impeded dimerization of hHsp90β.

Random mutation at positions 558 and 621 of hHsp90β

We finally tested whether Thr₅₆₆ and Ala₆₂₉ of hHsp90α are essential for the high-affinity interaction or if other amino acids could serve as substitutes. After the introduction of a random mutation at Ala₅₅₈ or Met₆₂₁ of hHsp90β, the colonies with an interaction between hHsp90α-MC and hHsp90β-MC with some amino acids at either position

Table 3 Effect of amino acid conversion from hHsp90 α to hHsp90 β on the dimeric interaction

pKT25	Amino acid		pUT18C	Activity (%) ^a
pKT25	—	—	hHsp90 α -MC	14.3 $\pm$ 7.0
hHsp90 α -M	Thr ₅₆₆	—	hHsp90 α -MC	100.0 $\pm$ 4.7 ^b
hHsp90 β -M	Ala ₅₅₈	—	hHsp90 α -MC	54.2 $\pm$ 11.2**
hHsp90 α -M	Ala ₅₆₆	—	hHsp90 α -MC	69.1 $\pm$ 24.2*
Thr566Ala				
pKT25	—	—	hHsp90 α -MC	9.7 $\pm$ 4.8
hHsp90 α -C	—	Ala ₆₂₉	hHsp90 α -MC	100.0 $\pm$ 6.2 ^b
hHsp90 β -C	—	Met ₆₂₁	hHsp90 α -MC	35.9 $\pm$ 2.3***
hHsp90 α -C	—	Met ₆₂₉	hHsp90 α -MC	54.9 $\pm$ 13.9**
Ala629Met				

Thr₅₆₆ and Ala₆₂₉ of hHsp90 α , respectively, are equivalent to Ala₅₅₈ and Met₆₂₁ of hHsp90 β . Thr₅₆₆ of hHsp90 α -M and Ala₆₂₉ of hHsp90 α -C were substituted to Ala and Met, respectively. ^aMean $\pm$ S.D. (n=3) ^bP-values were calculated in comparison with respective controls (100%). *P<0.05, **P<0.01, ***P<0.001

were grown up as red colonies on MacConkey agar plates. Their nucleotide sequencing analysis and activity measurement revealed that at Ala₅₅₈ of hHsp90 β , Val, Ile, and Arg substituted for the role of Thr₅₆₆ of hHsp90 α in the interaction with hHsp90 α -MC (Table 4). Tyr partially substituted for this role. The clone encoding Thr was not obtained. Thus, there was no apparent tendency on the amino acids that could substitute the role of Thr₅₆₆ of hHsp90 α . On the other hand, at Met₆₂₁ of hHsp90 β , the clone encoding Ala, being reversed to hHsp90 α , was obtained. Moreover, Trp and Val partially substituted for Ala₆₂₉ of hHsp90 α . Ser substituted for it less efficiently. Therefore, hydrophobic amino acids such as Trp and Val appeared to be preferably substituted for Ala₆₂₉. However, because Met₆₂₁ of hHsp90 β is also hydrophobic amino acid, hydrophobicity should not be the sole determinant of the amino acid at position 629 of hHsp90 α .

Discussion

The C-terminal 191 amino acids (residues 542–732) are responsible for the dimerization of hHsp90 (15). Among them, there are 28 amino acid substitutions occurring between two isoforms appeared to be involved in the impeded dimerization of hHsp90 β . Our previous study further narrowed the region to amino acids 542–697, in which there are 16 amino acid substitutions (Nemoto et al. 1995). The present study clearly demonstrated that five amino acids among the 16 substitutions were affected by the dimer formation of hHsp90 β (Table 2). Especially, two conversions, i.e., conversions from Thr₅₆₆ and Ala₆₂₉ of hHsp90 α to Ala and Met, respectively, predominantly impeded the dimerization of hHsp90 β and β isoforms

partially affected the dimerization. Furthermore, we demonstrated that Val, Ile, and Arg could substitute for Thr₅₆₆ and that Trp and Val partially substituted for Ala₆₂₉ on the dimeric interaction. This finding indicated the notion that Thr₅₆₆ as well as Ala₆₂₉ of hHsp90 α are not essential for the dimerization. In fact, random mutagenesis analysis revealed that several amino acids could substitute for Thr₅₆₆ and Ala₆₂₉ of hHsp90 α on the dimeric interaction. Nevertheless, it should be reinforced that the conversion from Thr₅₆₆ and Ala₆₂₉ of hHsp90 α to Ala₅₅₈ and Met₆₂₁ of hHsp90 β , respectively, affects the dimerization of Hsp90.

The dimer-forming activity of mouse Hsp84, mouse ortholog of hHsp90 β , is less potent than that of mouse Hsp86 (Minami et al. 1994). The 16 amino acids substituted between hHsp90 isoforms are completely conserved in mouse orthologs. There are four additional amino acid substitutions between the two mouse isoforms at amino acids 542–697, but they do not vary between the two human isoforms. Thus, we propose that the impeded dimerization of mouse Hsp84 is mediated by the mechanism identical to that of hHsp90 β .

The domain structures of human and yeast Hsp90 and the C-terminal structure of HtpG have been reported (Prodromou et al. 1997; Stebbins et al. 1997; Meyer et al. 2003). Although the full-length structure of human Hsp90 remains

Table 4 Amino acids at the positions of Ala₅₅₈ and Met₆₂₁ of hHsp90 β that substitute for the roles of Thr₅₆₆ and Ala₆₂₉ of hHsp90 α , respectively

pKT25	Nucleotides	Amino acid	pUT18C	Activity (%) ^a
pKT25			hHsp90 α -MC	8.6 $\pm$ 0.8
hHsp90 α -MC	(ACA	Thr ₅₆₆)	hHsp90 α -MC	100.0 $\pm$ 11.2**
hHsp90 β -MC	(GCA	Ala ₅₅₈)	hHsp90 α -MC	20.8 $\pm$ 3.8 ^b
hHsp90 β -MC	ACA	Thr ₅₅₈	hHsp90 α -MC	90.4 $\pm$ 6.3**
Random mutation				
hHsp90 β -MC	GTC	Val ₅₅₈	hHsp90 α -MC	91.9 $\pm$ 14.7*
	CGG	Arg ₅₅₈	hHsp90 α -MC	91.6 $\pm$ 5.1**
	ATT	Ile ₅₅₈	hHsp90 α -MC	88.7 $\pm$ 16.5*
	TAT	Tyr ₅₅₈	hHsp90 α -MC	60.5 $\pm$ 9.5*
pKT25			hHsp90 α -MC	8.7 $\pm$ 1.7
hHsp90 α -MC	(GCA	Ala ₆₂₉)	hHsp90 α -MC	100.0 $\pm$ 12.3**
hHsp90 β -MC	(ATG	Met ₆₂₁)	hHsp90 α -MC	26.4 $\pm$ 2.0 ^b
hHsp90 β -MC	GCA	Ala ₆₂₁	hHsp90 α -MC	86.5 $\pm$ 14.5*
Random mutation				
hHsp90 β -MC	GCA	Ala ₆₂₁	hHsp90 α -MC	90.7 $\pm$ 7.9**
	TGG	Trp ₆₂₁	hHsp90 α -MC	61.3 $\pm$ 9.2*
	GTG	Val ₆₂₁	hHsp90 α -MC	59.5 $\pm$ 11.9*
	TCG	Ser ₆₂₁	hHsp90 α -MC	46.3 $\pm$ 7.5*

Thr₅₆₆ and Ala₆₂₉ of hHsp90 α , respectively, are equivalent to Ala₅₅₈ and Met₆₂₁ of hHsp90 β .

^aMean $\pm$ SD, (n=3)

^bP-values were calculated in comparison with the values of (hHsp90 β -MC). *P<0.01, **P<0.001

unknown, the full-length structure of HtpG from *E. coli* has been already elucidated (Harris et al. 2004). The dimeric structure of HtpG undergoes dramatic nucleotide-dependent conformational rearrangement (Shiau et al. 2006). Thr₅₆₆ and Ala₆₂₉ of hHsp90 α are equivalent to Ala₅₀₇ and Glu₅₆₃ of *E. coli* HtpG, respectively. The region starting from Ala₅₀₁ of HtpG, which is equivalent to Lys₅₆₀ of hHsp90 α , forms a C-terminal core unit composed of four α -helices and three β -sheets. Hence, both of the two amino acids responsible for the impeded dimerization of hHsp90 β are located within this C-terminal unit, and are located at the surface of the unit. This conformation is also likely on hHsp90 α , because the Met₆₂₈–Ala₆₂₉ bond as well as the Tyr₆₂₇–Met₆₂₈ bond of hHsp90 α , was selectively cleaved by limited proteolysis with chymotrypsin (Nemoto et al. 1995). Thus, it is likely that the substitution of the two amino acids in hHsp90 β affects the interaction with the counterpart of an Hsp90 dimer. Although the possibility that the substitutions might affect the steric structure of the unit cannot be completely eliminated; their outer locations and their non-conservativeness among hHsp90 isoforms and other family members strongly suggest that the former was the case.

It has been reported that both of Hsp90 isoforms are associated with the glucocorticoid receptor (Mendel and Orti 1988), indicating that both isoforms as a molecular chaperone for the steroid receptor signal system. Hence, the physiological meaning of the impeded dimerization of Hsp90 β remains unknown. Even if there is a subtle difference in the chaperone activities between the two Hsp90 isoforms, it may be difficult to estimate it at present.

A single nucleotide polymorphism that causes a mis-sense mutation from glutamine to histidine at position 488 of Hsp α Hsp90 was observed in Caucasians (Passarino et al. 2003). Subsequently, a study using a yeast expression system proved that the mutated Hsp90 α severely reduced the growth of cells compared with that obtained with wild-type Hsp90 α (MacLean et al 2006). Our preliminary study indicated that the defect was caused by the impeded dimerization of mutant Hsp90 (T. Kobayakawa, S. Yamada, and T. K. Nemoto, unpublished observation). Thus, we currently suspect that the impeded dimerization of Hsp90 β is also related to the chaperone activity, if some.

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