Substitution of only two residues of human Hsp90α causes impeded dimerization of Hsp90β

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Running title

Amino acid residues responsible for the impeded dimerization of Hsp90β

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Abbreviations

Hsp, heat shock protein; Hsp90, 90-kDa heat shock protein; hHsp90, human Hsp90; hHsp90α and hHsp90β, 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; and SDS, sodium dodecyl sulfate.

Abstract Two isoforms of the 90-kDa heat shock protein (Hsp90), i.e., Hsp90α and Hsp90β, 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 Hsp90β (Nemoto T, Ohara-Nemoto Y, Ota M, Takagi T, Yokoyama K. 1995. *Eur J Biochem* **233:** 1-8). The present study was performed to define the amino acid substitutions that cause the impeded dimerization of Hsp90β. Bacterial two-hybrid analysis revealed that, among the 16 amino acids, the conversion from Ala_{558} of Hsp90β to Thr_{566} of Hsp90α and that from Met_{621} of Hsp90β to Ala_{629} of Hsp90α most efficiently reversed the dimeric interaction, and that the inverse changes from those of Hsp90α to Hsp90β primarily explained the impeded dimerization of Hsp90β. Taken together we conclude that the conversion of Thr_{566} and Ala_{629} of Hsp90α to Ala_{558} and Met_{621} primarily responsible for impeded dimerization of Hsp90β.

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 Finkelson 1984; Borkovich et al 1989). In mammalian cells, two isoforms of the cytosolic Hsp90, called Hsp90α (hHsp90α) and Hsp90β (hHsp90β) 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 hHsp90α and hHsp90β, respectively. The amino acid sequence of hHsp90α is 85.5% identical to that of hHsp90β. 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 of 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 hHsp90α (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 Hsp90β possess an impeded dimerization activity compared with the dimerization activity of Hsp90α (Minami et al 1991; Minami et al 1994;

Nemoto et al 1995). Among the C-terminal 191 amino acids of hHsp90α 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 hHsp90β (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 hHsp90β. 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 hHsp90α into 3 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 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.

hHsp90α and hHsp90β are composed of 732 and 724 amino acids, respectively. The difference is caused by the deletion of amino acids in hHsp90β corresponding to residues 9-13 and 241-243 of hHsp90α. Accordingly, Thr_{566} and Ala_{629} of hHsp90α, respectively, are equivalent to Ala_{558} and Met_{621} of hHsp90β.

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

Construction of plasmids encoding the full-length forms of hHsp90α (Nemoto *et al.*, 1996), hHsp90β, human Grp94 (Roi 1998), HtpG from *E. 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 μg/ml of ampicillin

and 25 μg/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 hHsp90β was amplified by PCR with sense primer 5'-CTTGGATCCATGCCTGAGGAAGTGCACCA and antisense primer 5'-ACTGTCGACCTAATCGACTTCTTC 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 μg/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^{o}C, recombinant proteins were expressed at 30^{o}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. Construction of pKT25-hHsp90α carrying the M domain [pKT25-hHsp90α-M (amino acids 401-618)], the MC domain [pKT25-hHsp90α-MC (amino acids 401-732)], and the C domain [pKT25-hHsp90α-C (amino acids 619-732)] and pUT18C-hHsp90α carrying MC [pUT18C-hHsp90α-MC] was described previously (Yamada et al 2003; Kawano et al 2004). For construction of pKT25-hHsp90β-MC and -C, the corresponding regions were amplified by PCR with appropriate primers, and then inserted in an *Xba*I-*Sma*I site of pKT25. For construction of pKT25-hHsp90β-M, the DNA fragment encoding the M domain was amplified by PCR with appropriate primers, and then,

inserted into a *Pst*I-*Bam*HI 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 ± S.D. 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-hHsp90β-MC were exchanged to the corresponding amino acids in the Hsp90α sequence by PCR-based site-directed mutagenesis in combination with *Dpn*I degradation of the template DNA (see Fig. 2). Inversely, Thr_{566} encoded by pKT25-hHsp90α-M, which was equivalent to Ala_{558} of hHsp90β, was substituted to Ala and Ala_{629} encoded by pKT25-hHsp90α-C, which was equivalent to Met_{621} of hHsp90β, was substituted to Met as described above.

Random mutation at Ala_{558} or Met_{621} was introduced into pKT25-hHsp90β-MC by PCR according to the method of Imai (27). For mutagenesis of nucleotides encoding Ala_{558}, 5'-NNNAAGTTTGAGAACCTCTGCAAGC-3' (N=A, G, T, or C) and 5'-CTTGCTCTCTTCCATCTTCTTCTTC-3' were used the respective 5'- and 3'-primers. For mutagenesis of nucleotides encoding Met_{621}, 5'-NNNGCCAAAAAGCACCTGGAGATCA-3' and 5'-CATATAGCCCATGGTGGAGTTGT-3' were used as 5' and 3' primers, respectively. Following PCR with pKT25-hHsp90β-MC as a template, the PCR fragments were blunt-ended, phosphorylated, and then ligated. BTH101[pUT18-hHsp90α-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.

In order 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 (50V) 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 Hsp90β are lower than those of their respective Hsp90α. However, it is not known whether the impeded dimerization is specific to mammalian Hsp90β. 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 hHsp90α (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 hHsp90β (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 hHsp90β (35 kDa in lane 2 and 38 kDa in lane 5) failed to be determined. Because hHsp90β 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 hHsp90β.

In order to examine their configurations, we separated the purified proteins on PAGE under non-denaturing conditions. As reported previously (Nemoto et al 1995), hHsp90α 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, hHsp90β 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 hHsp90β was

expressed with the second vector (lane 5). A band at the position equivalent to that of the hHsp90β 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 hHsp90β. These findings demonstrate that hHsp90β had the least potent dimerization activity among the family members tested in Fig. 1. In some preparations of hHsp90β, 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 hHsp90β 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 Hsp90β (Nemoto et al 1995), we decided to compare the activity of MC domain. Moreover, in order 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. Hsp90α-MC was used as a binding partner, because the bindings to Hsp90β-MC were much lower than those to Hsp90α-MC (data not shown). As shown in Table 1, hHsp90α-C efficiently bound to hHsp90α-MC and hHsp90β-C bound to it less efficiently. Moreover, the binding of hHsp90β-M to hHsp90α-MC was poor compared with that of hHsp90α-M. Therefore, the impeded dimerization was faithfully reproduced by the two-hybrid system. Table 1 also demonstrates that the impeded dimer-forming activity of hHsp90β 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 hHsp90α and hHsp90β (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 hHsp90β (Fig. 2, plus). 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 Ala_{618}-Leu_{619}, hence the first 9 amino acids belong to the M domain; and the remaining 7 to the C domain.

When 16 amino acids of MC domain of hHsp90β were substituted to those of hHsp90α, in two cases (Ala_{558} and $Met_{621}$of hHsp90β), there were significant increases in the interaction comparable to that of hHsp90α-MC (Table 2). Moderate enhancement was observed in three cases (Met_{553}, Ser_{586}, and Val_{656} 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 Thr_{566} and Ala_{629} of hHsp90α to Ala_{558} and Met_{621} 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. In order to evaluate the significance of the amino acid substitution at the two positions, we further 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-M Ala_{566} to hHsp90α-MC was decreased to that comparable to that of hHsp90β-M (Table 3). The activity of hHsp90α-C Met_{629} 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 Thr_{566} and Ala_{629} of hHsp90α are essential for the high affinity interaction or if other amino acids could serve as substitutes. Following the introduction of a random mutation at Ala_{558} or Met_{621} of hHsp90β, the colonies with an interaction between hHsp90α-MC and hHsp90β-MC with some amino acids at either position were grown up as red colonies on MacConkey agar plates. Their nucleotide sequencing analysis and activity measurement revealed that, at Ala_{558} of hHsp90β, Val, Ile and Arg substituted for the role of Thr_{566} of hHsp90α in the interaction with hHsp90α-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 Thr_{566} of hHsp90α. On the other hand, at Met_{621} of hHsp90β, the clone encoding Ala, being reversed to hHsp90α, was obtained. Moreover, Trp and Val partially substituted for Ala_{629} of hHsp90α. Ser substituted for it less efficiently. Therefore, hydrophobic amino acids, such as Trp and Val, appeared to be preferably substituted for Ala_{629}. However, because Met_{621} of hHsp90β is also hydrophobic amino acid, hydrophobicity should not be the sole determinant of the amino acid at position 629 of hHsp90α.

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 hHsp90β. 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 affected of the dimer formation of hHsp90β (Table 2). Especially, two conversions, i.e., conversions from Thr_{566} and Ala_{629} of hHsp90α to Ala and Met, respectively, predominantly impeded the dimerization of hHsp90β. However, this does not imply that these two amino acids are most important for the dimerization. For instance,

we previously demonstrated that a highly hydrophobic segment of hHsp90α (amino acids 662-672) is essential for the dimerization (Yamada et al 2003). To the contrary, the conversion of the two amino acids between the α and β isoforms partially affected the dimerization. Furthermore, we demonstrated that Val, Ile, and Arg could substitute for Thr_{566} and that Trp and Val partially substituted for Ala_{629} on the dimeric interaction. This finding indicated the notion that Thr_{566} as well as Ala_{629} of hHsp90α are not essential for the dimerization. In fact, random mutagenesis analysis revealed that several amino acids could substitute for Thr_{566} and Ala_{629} of hHsp90α on the dimeric interaction. Nevertheless, it should be reinforced that the conversion from Thr_{566} and Ala_{629} of hHsp90α to Ala_{558} and Met_{621} of hHsp90β, respectively, affects the dimerization of Hsp90.

The dimer-forming activity of mouse Hsp84, mouse ortholog of hHsp90β 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 4 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 hHsp90β.

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 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. Thr_{566} and Ala_{629} of hHsp90α are equivalent to Ala507 and Glu_{563} of *E. coli* HtpG, respectively. The region starting from Ala_{501} of HtpG which is equivalent to Lys_{560} of hHsp90α 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 hHsp90β are located within this C-terminal unit, and are located at the surface of the unit. This conformation is also likely on hHsp90α, because the Met_{628}-Ala_{629} bond as well as Tyr_{627}-Met_{628} bond of hHsp90α 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 hHsp90β affects the interaction with the counterpart of an Hsp90 dimer. Although the possibility that the substitutions might affect the steric structure of the unit can not 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, physiological meaning of the impeded dimerization of Hsp90β 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 missense mutation from glutamine to histidine at position 488 of Hspα Hsp90 was observed in Caucasians (Passarino et al 2003). Subsequently, a study using a yeast expression system proved that the mutated Hsp90α severely reduced the growth of cells compared with that obtained with wild-type Hsp90α (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 TK Nemoto, unpublished observation). Thus, we currently suspect that the impeded dimerization of Hsp90β is also related to the chaperone activity, if some.

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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 ± S.D. (n=4) *p<0.001 *P-values* were calculated in comparison with respective controls (hHsp90α).

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

	pKT25-	Substitution From	To	pUT18C-	Activity (%)[a.]
	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, Met_{553} of hHsp90β is equivalent to Gln_{561} of hHsp90α and so on.

[a]Mean ± S.D. (n=5) [b]*P-values* were calculated in comparison with the value of hHsp90β-MC. *p<0.05, **p<0.01, ***p<0.001

Table 3. Effect of amino acid conversion from hHsp90α to hHsp90β on the dimeric interaction.

pKT25-	Amino acid		pUT18C-	Activity (%)[a]
pKT25	-	-	hHsp90α-MC	14.3 ± 7.0
hHsp90α-M	Thr_{566}	-	hHsp90α-MC	100.0 ± 4.7[b]
hHsp90β-M	Ala_{558}	-	hHsp90α-MC	54.2 ± 11.2**
hHsp90α-M Thr566Ala	Ala_{566}	-	hHsp90α-MC	69.1 ± 24.2*
pKT25	-	-	hHsp90α-MC	9.7 ± 4.8
hHsp90α-C	-	Ala_{629}	hHsp90α-MC	100.0 ± 6.2[b]
hHsp90β-C	-	Met_{621}	hHsp90α-MC	35.9 ± 2.3***
hHsp90α-C Ala629Met	-	Met_{629}	hHsp90α-MC	54.9 ± 13.9**

Thr_{566} and Ala_{629} of hHsp90α, respectively, are equivalent to Ala_{558} and Met_{621} of hHsp90β. Thr_{566} of hHsp90α-M and Ala_{629} of hHsp90α-C were substituted to Ala and Met, respectively. [a]Mean ± S.D. (n=3) [b]*P-values* were calculated in comparison with respective controls (100%). *P<0.05, **P<0.01, ***P<0.001

Table 4. Amino acids at the positions of Ala_{558} and Met_{621} of hHsp90β that substitute for the roles of Thr_{566} and Ala_{629} of hHsp90α, respectively.

pKT25-	Nucleotides	Amino acid	pUT18C-	Activity (%)[a]
pKT25			hHsp90α-MC	8.6 ± 0.8
hHsp90α-MC	(ACA	Thr_{566})	hHsp90α-MC	100.0 ± 11.2**
hHsp90β-MC	(GCA	Ala_{558})	hHsp90α-MC	20.8 ± 3.8[b]
hHsp90β-MC	ACA	Thr_{558}	hHsp90α-MC	90.4± 6.3**
Random mutation				
hHsp90β-MC	GTC	Val_{558}	hHsp90α-MC	91.9 ± 14.7*
	CGG	Arg_{558}	hHsp90α-MC	91.6 ± 5.1**
	ATT	Ile_{558}	hHsp90α-MC	88.7± 16.5*
	TAT	Tyr_{558}	hHsp90α-MC	60.5 ± 9.5*
pKT25			hHsp90α-MC	8.7 ± 1.7
hHsp90α-MC	(GCA	Ala_{629})	hHsp90α-MC	100.0 ± 12.3**
hHsp90β-MC	(ATG	Met_{621})	hHsp90α-MC	26.4 ± 2.0[b]
hHsp90β-MC	GCA	Ala_{621}	hHsp90α-MC	86.5± 14.5*
Random mutation				
hHsp90β-MC	GCA	Ala_{621}	hHsp90α-MC	90.7 ± 7.9**
	TGG	Trp_{621}	hHsp90α-MC	61.3± 9.2*
	GTG	Val_{621}	hHsp90α-MC	59.5 ± 11.9*
	TCG	Ser_{621}	hHsp90α-MC	46.3 ± 7.5*

Thr_{566} and Ala_{629} of hHsp90α, respectively, are equivalent to Ala_{558} and Met_{621} of hHsp90β.

[a]Mean ± S.D. (n=3) [b]*P-values* were calculated in comparison with the values of (hHsp90β-MC). *P<0.01, **P<0.001

FIGURE LEGENDS

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, hHsp90α; lane 2, hHsp90β; lane 3, human Grp94; lane 4, HtpG from *E. coli*; lane 5, hHsp90β; lane 6, human Trap1; lane 7, HtpG from *P. gingivalis*; and lane M, molecular marker markers. Purified proteins (0.5 and 1 μg proteins, respectively) were loaded onto the gel in panels "**A**" and "**B**."

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 Ala_{618}-Leu_{619}.

Fig. 1

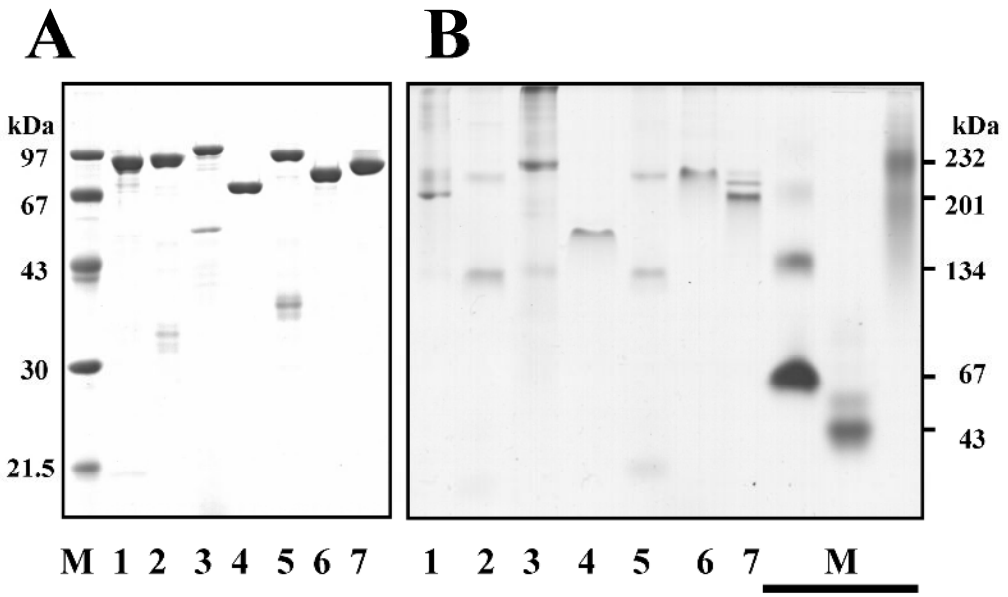
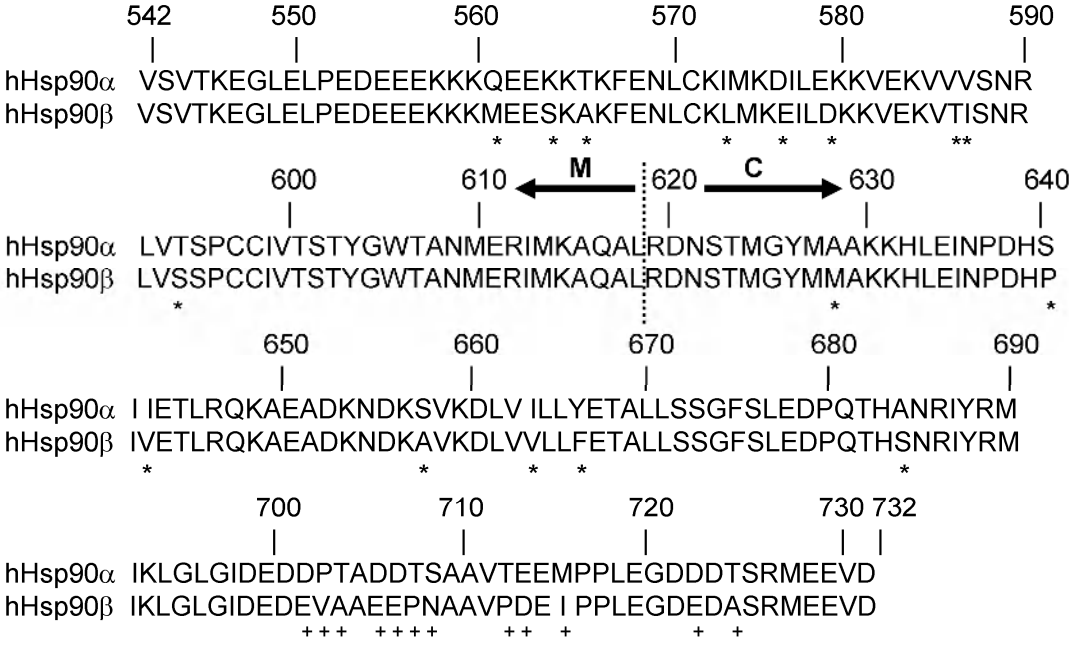

A
B
kDa
97
67
43
30
21.5
M 1 2 3 4 5 6 7
1 2 3 4 5 6 7 M
kDa
232
201
134
67
43

Fig. 2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600 610 M 620 C 630 640
hHsp90α LVTSPCCIVTSTYGWTANMERIMKAQALRDNSTMGYMAAKKHLEINPDHS
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hHsp90β IVETLRQKAEADKNDKAVKDLVVLLFETALLSSGFSLEDPQTHSNRIYRM
700 710 720 730 732
hHsp90α IKLGLGIDEDDPTADDTSAAVTEEMPPLEGDDDTSRMEEVD
hHsp90β IKLGLGIDEDEVAAEEPNAAVPDE I PPLEGDEDASRMEEVD