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Hydrophobic Segment within the C-terminal Domain Is Essential for Both Client-Binding and Dimer Formation of the HSP90-Family Molecular Chaperone

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SUMMARY

The α isoform of human 90-kDa heat shock protein (HSP90 α) is composed of 3 domains: the N-terminal (residues 1-400); middle (residues 401-615) and C-terminal (residues 621-732). The middle domain is simultaneously associated with the N- and C-terminal domains, and the interaction with the latter mediates the dimeric configuration of HSP90. Besides one in the N-terminal domain, an additional client-binding site exists in the C-terminal domain of HSP90. The aim of the present study is to elucidate the regions within the C-terminal domain responsible for the bindings to the middle domain and to a client protein, and to define the relationship between the 2 functions. A bacterial 2-hybrid system revealed that residues 650-697 of HSP90 α were essential for the binding to the middle domain. An almost identical region (residues 657-720) was required for the suppression of heat-induced aggregation of citrate synthase, a model client protein. Replacement of either Leu665-Leu666 or Leu671-Leu672 to Ser-Ser within the hydrophobic segment (residues 662-678) of the C-terminal domain caused the loss of bindings to both the middle domain and the client protein. The interaction between the middle and C-terminal domains was also found in human 94-kDa glucose-regulated protein. Moreover, *Escherichia coli* HtpG, a bacterial HSP90 homologue, formed hetero-dimeric complexes with HSP90 α and the 94-kDa glucose-regulated protein through their middle-C-terminal domains. Taken together, it is concluded that the identical region including the hydrophobic segment of the C-terminal domain is essential for both the client binding and dimer formation of the HSP90-family molecular chaperone and that the dimeric configuration appears to be similar in the HSP90-family proteins.

INTRODUCTION

The 90-kDa heat shock protein (HSP90)¹ is a ubiquitously distributed molecular chaperone and is an essential protein in eukaryotic cells [1]. Most, if not all, compartments of mammalian cells contain specific members of HSP90. For instance, 2 HSP90 isoforms, HSP90 α [2] and HSP90 β [3], are present in the cytosol; the 94-kDa glucose-regulated protein (GRP94/gp96) is expressed in the lumen of endoplasmic reticulum [4]; and TRAP1/hsp75 is expressed in mitochondria [5]. Also, HtpG exists in prokaryotic cells [6], although its expression is not essential for the organisms [7, 8].

HSP90 is either transiently or stably associated with specific client proteins that are unstable unless chaperoned with HSP90. Various regions of HSP90 have been proposed to be involved in the interactions with such target proteins. For instance, a highly charged region of chick HSP90 (amino acids 221-290) is essential for the binding to estrogen and mineralocorticoid receptors [9]; and this region is also involved in the binding to the α subunit of casein kinase CK2 [10]. However, the corresponding highly charged region and C-terminal 35 residues that are specific to mammalian HSP90 can be deleted from yeast HSP82 [11]. Serial deletion experiments on HSP90 β demonstrated that amino acids 327-340, which are distinct but proximal to the charged region, are essential for chaperoning of serine/threonine kinase Akt/PKB [12]. Two separate regions were proposed to be involved in the binding to the progesterone receptor [13]. At present, it is ambiguous whether this discrepancy is caused by the variation in the binding sites of HSP90 for the respective substrates or if the respective regions are responsible for certain aspects of the chaperoning mechanism.

Another approach by use of model client proteins has been employed to clarify the client-binding sites of HSP90. By use of citrate synthase (CS) and insulin, it was reported that mammalian HSP90 possesses 2 distinct client-binding sites [14, 15]: one of them is located in the N-terminal domain and its activity is modulated by ATP and

geldanamycin, a specific inhibitor of HSP90 molecular chaperone; and the other is in the C-terminal domain. Minami *et al.* [16] and Tanaka *et al.* [17] confirmed the existence of these respective client-binding sites in the N- and C-terminal domains. Similarly, the C-terminal fragment (residues 494-782 as a mature form) of human GRP94 protects the catalytic subunit of protein kinase CK2 against thermal aggregation [18]. In contrast, we found a single client-binding site in *E. coli* HtpG, which was localized solely in the N-terminal domain (residues 1-336) of the 624-amino acid protein [17].

All members of the HSP90 family proteins so far studied exist as dimers [19-22]. Exceptionally, although HSP90 β that was analyzed by polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions predominantly existed as a monomer [19], it still existed as a dimer and oligomers in rat liver cytosol, but tended to dissociate into monomers under the electrophoretic conditions [23]. Disruption of the dimeric structure of HSP90 is lethal in yeast [24], although some of the monomeric mutants of HSP90 are able to confer viability and interact with the estrogen receptor [25].

The C-terminal 49 amino acids are essential for the dimer formation of HSP90 [24] and 191 amino acids are sufficient for the function [20]. We previously proposed on human HSP90 α [20] and *E. coli* HtpG [26] that they form a dimer in an anti-parallel fashion through a pair of the interactions between the middle domain and the C-terminal domain. Similarly, the C-terminal 326 amino acids of barley GRP94 [22] and 200 amino acids of canine GRP94 [27] are sufficient for the dimer formation. However, Wearsch and Nicchitta [27] proposed a distinct mechanism of dimer formation, on which the hydrophobic segment localized in the C-terminal domain interacts with each other.

In the present study, we investigated 2 issues with respect to the C-terminal domain of HSP90. One was the identification of the minimal essential region required for the interaction with the middle domain, which mediates the dimerization of HSP90, and the other, the identification of the minimal region of the C-terminal domain for the client binding. Taking in mind the fact that the 35-amino acid residues corresponding to the C-terminus of HSP90 are deleted in HtpG, we postulated that the regions within the C-terminal domain responsible for dimerization, *i.e.*, an interaction with the middle domain, and client binding, could be separated into the N- and C-terminal parts, respectively. However, the present study demonstrates that the 2 regions are unable to be separated and that the 2 functions are closely related to each other. We also reinvestigated the mode of dimer formation in the HSP90-family proteins.

EXPERIMENTAL PROCEDURES

Materials - Expression vector pQE9 and plasmid pREP4 were purchased from Qiagen Inc. (Chatsworth, CA, U.S.A.); and expression vector pGEX4T-1, glutathione-Sepharose and low-molecular-weight markers, from Amersham Pharmacia Biotech (Uppsala, Sweden). Talon metal affinity resin was obtained from Clontech Laboratories Inc. (Palo Alto, CA, U.S.A.). Porcine heart citrate synthase (CS) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). All other reagents were of analytical grade.

Plasmid construction

DNA fragments carrying truncated forms of human HSP90 α amplified by PCR and cut with *Bam*HI and *Sal*I, were inserted into a *Bam*HI/*Sal*I site of pQE9 (designated pH₆HSP90 α). Construction of truncated forms of pH₆HSP90 α , *i.e.*, pH₆HSP90 α 542-732, 542-728, 542-720, 542-697 and 542-687 was described previously [28]. Truncated forms of HSP90 α were also expressed as glutathione *S*-transferase (GST)-fusion proteins. The DNA fragments encoding HSP90 α 657-732, 676-732 and 697-732 were amplified by PCR and inserted into a *Bam*HI/*Sal*I site of pGEX-4T-1. Construction of the plasmid encoding amino acids 1-43/604-732 was described previously [28].

We also expressed the middle and C-terminal domains of human GRP94 as GST-fusion proteins. Although the domain structures and the domain boundaries of human GRP94 have not been determined, we tentatively defined the boundary between the N-terminal and middle domains to be Arg427-Glu428 and that between the middle and C-terminal domains to be Lys650-Asp651 by comparison with the amino acid sequence of human GRP94 [29] with those of human HSP90 α [28] and *E. coli* HtpG [26]. Amino acid numbers refer to those of the mature form: Accordingly, the initial Met in the prepeptide corresponds to -21 and the mature form corresponds to Asp1-Leu782. The DNA fragments encoding the middle domain (Glu428-Lys650)

and the C-terminal domain (Asp651-Leu782) of human GRP94 [29] were amplified by PCR and then inserted into a *Bam*HI/*Sal*I site of pGEX4T-1 (designated pGST-GRP94-M and pGST-GRP94-C, respectively). Y1090 cells transformed with these plasmids were selected on Luria broth agar containing 50 µg/ml of ampicillin. Constructed plasmids were verified by DNA sequencing.

Expression and purification of recombinant proteins

A histidine hexamer-tagged form of recombinant proteins was expressed and purified by use of Talon affinity resin according to the manufacturer's protocol, except that 10 mM imidazole was added to the lysis/washing buffer. Bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol. GST-fusion proteins were expressed and purified by binding to glutathione-Sepharose as described previously [30].

Bacterial 2-hybrid system

Bacterial strain BTH101 [*F*⁻, *cya-99*, *araD139*, *gal15*, *galK16*, *rpsL1 (StrI^r)*, *hsdR2*, *mcrA1*, *mcrB1*] and plasmids pKT25^{kan} and pUT18C^{amp} were provided by Drs. D. Ladant (Pasteur Institute, Paris, France) and L. Selig (Hybrigenics, S. A., Paris, France). An improved version of the bacterial 2-hybrid system [31] was employed to evaluate domain-domain interactions of HSP90, GRP94 and HtpG. This method is based on the interaction-mediated reconstitution of an adenylate cyclase activity in the enzyme-deficient *E. coli* strain, BTH101. Because human HSP90α was proteolysed at Lys615-Ala616 and Arg620-Ala621 by trypsin [20, 28], the border between the middle and C-terminal domains in the present study was set to Lys618-Leu619. The PCR fragment carrying the middle-C-terminal domains (residues 401-732) of HSP90α was inserted in a *Pst*I/*Bam*HI site of pUT18C^{amp}. The DNA fragments carrying the C-terminal domain (residues 619-732) of HSP90α or its truncated forms amplified by PCR were inserted into a *Pst*I/*Bam*HI site of pKT25^{kan}. The DNA fragments encoding

tentative middle domain and C-terminal one of human GRP94 were amplified by PCR and then inserted in an *XbaI/BamHI* site of both pUT18C^{amp} and pKT25^{kan}. The DNA fragment encoding the middle-C-terminal domains of GRP94 was amplified by PCR and then inserted in an *XbaI/BamHI* site of pUT18C^{amp}. The construction of pKT25^{kan}-HtpG 337-624/the middle-C-terminal domains was described previously [17].

An *E. coli* strain BTH101 was co-transformed with pUT18C^{amp}- and pKT25^{kan}-derived plasmids. The extent of reconstitution of the catalytic domains of *Bordetella pertussis* adenylate cyclase through the fused portions was quantified as β -galactosidase activity, which was measured after the bacteria had been cultured overnight at 30°C in Luria broth medium containing 50 μ g/ml of ampicillin and 25 μ g/ml kanamycin in the presence of 0.5 mM isopropyl- β -D-thiogalactopyranoside [31].

Suppression assay for heat-induced aggregation of CS

Heat-induced aggregation of CS and its suppression in the presence of recombinant proteins were measured as described previously [32]. In brief, CS (8 μ g) in the presence or absence of 6-48 μ g of recombinant proteins in 0.4 ml of 40 mM HEPES, pH 7.4, was transferred at 45°C. The absorbance at 360 nm was measured at 80 min, when the turbidity reached a plateau.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed at a polyacrylamide concentration of 12.5% in the presence of 0.1% SDS. Separated proteins were stained with Coomassie Brilliant Blue. Low-molecular-weight markers (Amersham Pharmacia BioTech, Uppsala, Sweden) were used as references.

Protein concentration

Protein concentrations were determined by the bicinchoninic acid method (Pierce,

Rockford, IL, U.S.A.).

RESULTS

Minimal region of the C-terminal domain sufficient for dimerization with the middle domain

The C-terminus of the C-terminal domain (Leu619-Asp732) of human HSP90 α was serially truncated, and the binding activity to the middle domain (Glu401-Lys618) was quantified by using the bacterial 2-hybrid system (Fig. 1). As reported previously on human HSP90 α [17], because the C-terminal domain could not associate with the middle domain, but associated with the middle-C-terminal domains, we used the middle-C-terminal domains as a binding partner of the C-terminal domain in the 2-hybrid system. As a result, H₆HSP90 α 619-728, 619-720 and 619-707 bound to the partner. Even H₆HSP90 α 619-697 possessed 72.5% of the maximal binding. However, truncation by additional 10 amino acids resulted in loss most of the binding. Thus, the C-terminal 35 amino acids of HSP90 α were dispensable without significant loss of the dimer-forming activity, whereas further 10-amino acid truncation disrupted the function.

Fig. 1

In turn, the N-terminal side of the C-terminal domain was truncated. Residues 629-732 as well as 619-732 (the C-terminal domain) had the binding activity (Fig. 1). It should be noted that the full-length form of HSP90 α was cleaved with chymotrypsin at Tyr627-Met628 and Met628-Ala629 bonds [20]. Thus, the Ala616-Met628 segment may not be essential for the function of the C-terminal domain. Hence, it was reasonable that residues 629-732 still retained the binding activity. The binding activity of residues 650-732, *i.e.*, a further deletion of the N-terminus up to Lys649, was 48.5% of the positive control (Fig. 1). Thus, residues 650-732 were essential for the binding, although its N-terminal proximal site (residues 629-649) may also be involved in the association.

Fig. 2

Minimal region sufficient for the binding to a model client protein

Next, we measured the client-binding activity of the C-terminal site. We started from H₆HSP90α542-732, an N-terminally histidine hexamer-tagged form, for the C-terminal truncation experiment (Fig. 2a). The truncated proteins were purified to near homogeneity (Fig. 2b, lanes 1-3 and lane 5) with an exception of GST-HSP90α 542-697 (lane 4). Aggregation of CS induced at 45°C was suppressed in the presence of H₆HSP90α542-732 in a dose dependent manner. Its C-terminal truncation forms, *i.e.*, H₆HSP90α542-728 suppressed the CS aggregation (Fig. 2c). H₆HSP90α542-720 still suppressed the aggregation, but the efficiency appeared to be lower than those of H₆HSP90α542-732 and H₆HSP90α542-728. A further truncated form, HSP90α542-687, showed no suppression. We could not test whether or not GST-HSP90α542-697 would suppress the aggregation of CS, because the preparation contained doublet bands (Fig. 2b, lane 4) and self-aggregated at 45°C even in the absence of CS (data not shown).

We attempted to express even smaller fragments of N-terminal truncation than those of the C-terminal truncations. However, recombinant proteins were not quantitatively recovered in the expression system presumably due to the instability of exogenous proteins with small molecular masses in *E. coli*. Accordingly, the N-terminal-truncated forms were expressed as GST-fusion proteins with a relatively large moiety (Fig. 2a and b, 6-9). As shown in Fig. 2d, GST-HSP90α1-43/604-732 and GST-HSP90α657-732 could suppress the aggregation of the client protein. However, GST-HSP90α697-732 and GST-HSPα676-732, as well as GST, did not affect the process. Taken together, the data indicated that residues 657-720 were indispensable for the client-binding function. The activities of GST-fusion proteins

were consistently higher than those of the histidine-tagged forms (compare Fig. 2c and d), which may be related to the dimeric nature of GST-fusion proteins as reported previously [33]. The dimeric form may more efficiently bind to a client protein like a clamp, as proposed for the mechanism of the action of the N-terminal domain of HSP90 [34-36].

Fig. 3 and Table 1

Effect of amino acid replacements within the hydrophobic segment – The above findings revealed an overlap or even identity between the region (residues 650-697) required for the dimer formation and that (residues 657-720) for the binding to a client protein. Notably, a hydrophobic segment (residues 662-678) is located in the region (Fig. 3a). It is well known that high ionic strength does not induce the dissociation of an HSP90 dimer. Thereby, it is reasonably postulated that the hydrophobic segment is involved in dimeric interaction, and presumably in client binding as well. In fact, Wearsch and Nicchitta [27] previously proposed that 45 amino acids carrying this hydrophobic segment were sufficient for the dimerization of GRP94. Hence, on the C-terminal domain of HSP90 α , we substituted Leu665-Leu666 or Leu671-Leu672 located in this segment to Ser-Ser (Fig. 3a). As shown in Table 1, the C-terminal domain with either of these mutations completely lost its activity to bind to the middle-C-terminal domains.

HSP90 α 657-732 with substitutions as represented in Fig. 3a was also expressed as GST-fusion proteins (Fig. 3b), and the suppression on CS aggregation at an elevated temperature was tested. The substitutions caused the loss or a dose-dependent reduction in the suppression activity (Fig. 3c).

Table 2

Reinvestigation of the mode of dimer formation of GRP94

Because the C-terminal 326 residues of barley GRP94 [22] and 200 residues of canine GRP94 [27] are sufficient for the dimer formation, it is reasonable to postulate that the mode of the dimer formation is common among the HSP90-family proteins. However, it was reported that the 45 amino acids carrying the hydrophobic segment (see Fig. 3a) could self-dimerize when expressed as a fusion protein with a maltose-binding protein [27]. This configuration of a GRP94 dimer is apparently distinct from our dimer model on HSP90, in which the middle domain is associated with the C-terminal domain in an anti-parallel fashion [20, 37]. This phenomenon reminded us the finding that purified HSP90, GRP94 and HtpG self-oligomerize at elevated temperatures and that the phenomenon is closely related to the client-binding function of the proteins [32, 38]. Taken together, we assumed that the complex formation of the region carrying the hydrophobic segment of GRP94 is mediated via its client-binding activity. To settle this issue, we reinvestigated the domain-domain interaction of human GRP94 by use of the bacterial 2-hybrid system.

Table 2 shows that the dimerization was mediated via the interaction between the middle domain and the C-terminal one. Hence, we conclude that the C-terminal domain, which contains the hydrophobic region, does not associate with each other.

Table 3

Above findings let us further examine the possibility that hybrid dimers could be formed among 3 HSP90-family proteins. In a control experiment, the 2-hybrid experiment demonstrated homodimer formation of the middle-C-terminal domains of HSP90 α (Table 3). The 2-hybrid experiment by use of the middle-C-terminal domains showed the hetero-dimer formation between HSP90 α and HtpG and between GRP94

and HtpG. On the other hand, the complex was not formed between HSP90 α and GRP94 as reported previously [22].

Fig. 4

We finally investigated whether the client-binding site of GRP94 is localized in either the middle domain or the C-terminal one. GRP94-M and GRP94-C were expressed as GST-fusion proteins. They were purified to near homogeneity, although the preparation of GST-GRP94-M contained some amounts of 29-kDa GST species (Fig. 4a, lane 1). Fig. 4b clearly demonstrated that GST-GRP94-C suppressed the aggregation of CS at 45°C, but that GST-GRP94-M did not.

DISCUSSION

Several biochemical properties and the roles have been characterized on the C-terminal domain of HSP90. At first, the C-terminal pentapeptide of HSP90 was recognized by the tetra-tricopeptide repeat (TPR)-domain containing co-chaperone Hop, which links HSP90 to the HSP70-family proteins [39]. Secondly, residues 702-716 adjacent to the C-terminus form one of the 2 most immunogenic regions [28], which strongly suggests that this region is exposed at the outer surface of an HSP90 dimer. Thirdly, the C-terminal 49 amino acids are essential for the dimer formation [24]. Fourthly, the C-terminal domain of HSP90 contains a client-binding site with characteristics distinct from those of the site located at the N-terminal domain [14-17]. This C-terminal client-binding site also exists in GRP94 [40], but not in HtpG [17]. However, the respective studies dealt with one of these properties, and therefore, it is still ambiguous whether the regions, especially the region responsible for dimer formation and that for client binding, exist at distinct sites of the C-terminal region, or they are closely related to each other.

In our approach we initially focused on the C-terminal 35 amino acids of HSP90, of which the equivalent region is deleted in HtpG. Our hypothesis that the C-terminal 35 amino acids were not essential for the dimerization was verified by the data shown in Fig. 1. On the other hand, the second assumption that the 35 amino acids were involved in the client binding was not true, but the central part of the C-terminal domain, residues 657-720, was shown to be essential. Therefore, the 2 regions that were sufficient for both functions overlapped or were indistinguishable from each other. Their close relationship was ascertained by amino acid substitutions in the hydrophobic segment (Fig. 3 and Table 1).

Fig. 5

The present study demonstrated that, in HSP90 α , double mutations of Leu to Ser at positions 665 and 666 or 671 and 672 in the hydrophobic segment diminished or completely destroyed the client-binding and dimer-forming activities simultaneously. The amino acid sequence of the hydrophobic segment of HSP90 α was relatively conserved with those of human GRP94 and *E. coli* HtpG (Fig. 3a). However, the difference was evident by the hydropathy plot of the C-terminal domain according to Kyte and Doolittle [41]. As shown in Fig. 5, the corresponding region of HtpG is less hydrophobic, which may explain the incapability of the binding of the C-terminal domain of HtpG to a client protein [17].

We critically reviewed the previous study that demonstrated the dimer formation of the hydrophobic segment of GRP94 [27]: The maltose-binding protein-fused GRP94 segment migrated with a wide range of apparent molecular masses on a size-exclusion chromatography column, which indicated the formation of oligomers larger than a dimer. The present study on GRP94 demonstrated a direct interaction between the middle

domain and the C-terminal one, and that neither the C-terminal domain nor the middle domain homo-dimerized. Accordingly, we propose that the dimerization of the HSP90-family protein is generally achieved through a pair of heteromeric interactions between the middle and C-terminal domains. Self-oligomer formation of the hydrophobic segment of GRP94 [27] may reflect its potent client-binding capacity located in the C-terminal domain.

The perfect dimer configuration of the HSP90-family protein seems to be accomplished through a pair of the inter-molecular interactions between the middle and C-terminal domains as proposed previously [20], even if a single interaction between the middle and C-terminal domains might be sufficient to maintain the complex under the experimental conditions. Taken in mind the finding that the hybrid formation of the N-terminal and middle domains between human HSP90 α and *E. coli* HtpG [17], the conformational similarity of the HSP90-family proteins can be expanded to all domains of the protein.

It should be reminded that bacterial 2-hybrid experiments demonstrated the interaction between the middle and C-terminal domains of GRP94 (Table 2) as well as those of HtpG [17]. To the contrary, the combination failed to form a complex in HSP90 α [17], but the combination of the middle-C-terminal domains either with the middle domain or the C-terminal domain is required for the interaction (Fig. 1 and [17]). Presumably, the fine mode of the dimeric structure may be not identical among all members of the HSP90-family protein. Additionally, it should be noted that this phenomenon made it difficult to reconstitute the complex between the middle and C-terminal domains with purified samples *in vitro*, because HSP90 α -MC formed a stable dimer, with which neither middle domain nor C-terminal one added afterward was replaced (data not shown). Accordingly, an attempt to reconstitute such complex of HSP90 α *in vitro* was not successful (data not shown)

The importance of the C-terminal region for the HSP90 molecular chaperone has been indicated by Sullivan and Toft [13]: two separate regions of chicken HSP90 β (amino acids 381-441 and 601-677) are particularly important for the binding of the progesterone receptor. Hartson *et al.* [42] also proposed that a specific region near residue 600 determines the mode by which HSP90 interacts with substrates. Moreover, Glu651-Ile698 of human HSP90 α , which carries the hydrophobic segment, is required for activation of basic helix-loop-helix-helix (bHLH) protein, such as MyoD and E12 [43]. The findings in the present study on the client binding are consistent to these reports.

Human GRP94 and mouse HSP90 were identified as tumor-specific antigens expressed on the surface of various tumor cells [44, 45]. Recently, the C-terminal site of GRP94 bound to a vesicular stomatitis virus capsid-derived peptide was attributed to a charged region, Lys602-Asp-Lys-Ala-Leu-Lys-Asp-Lys609, by a photoaffinity labeling experiment [40]. This region is located in the middle domain (Glu428-Lys650), not in the C-terminal domain (Asp651-Leu782), in contrast to the results in the present study. At present, it remains unknown why this discrepancy occurred, but the dimer topology of the family proteins may provide a hint. That is, the middle domain associates with the C-terminal domain in a GRP94 dimer, and accordingly, the charged region of the middle domain may be adjacent to the hydrophobic segment of Domain C in the tertiary structure in a dimer. Therefore, it should be settled whether the Lys602-Lys609 charged region is truly indispensable for the client binding or simply present adjacent to the client-binding site to be affinity-labeled with the client peptide. This issue is now under investigation in our laboratory.

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FOOTNOTE

¹*Abbreviations:* HSP90, the 90-kDa heat shock protein; HSP90 α and HSP90 β , the α and β isoforms of HSP90, respectively; HtpG, an *E. coli* homologue of mammalian HSP90; GRP94, the 94-kDa glucose-regulated protein; GST, glutathione S-transferase; GST-HSP90 α and H₆HSP90 α , HSP90 α tagged with GST and a histidine hexamer (MRGSH₆GS), respectively, at the N-terminus; CS, citrate synthase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

FIGURE LEGENDS

Fig. 1. Minimal region of the C-terminal domain that is required for the dimerization. (a) The truncated forms of the C-terminal domain (residues 619-732) of human HSP90 α were expressed in combination with the middle-C-terminal domains (residues 401-732). Residues 662-678 constitute the hydrophobic segment (see Fig. 3a). Residues 698-732 correspond to the deleted region in *E. coli* HtpG. The extent of the association was estimated by the β -galactosidase activity. The value of the combination of intact C-terminal domain (residues 619-732) with the middle-C-terminal domains was set to 100%. Values are means \pm S.D. of 3 samples.

Fig. 2. Suppression of the heat-induced aggregation of CS by the C-terminal regions of HSP90 α . (a) HSP90 α 542-732 and its C-terminally truncated forms were expressed with an N-terminal histidine hexamer tag. HSP90 α 657-732, 676-732 and 697-732 were expressed as GST-fusion proteins. A dotted line indicates the boundary between the middle and C-terminal domains. (b) Purified proteins (1 μ g) were electrophoresed on SDS-PAGE gels. Lane numbers are identical to those in Fig. 2a. M, low-molecular-weight markers. (c and d) The increase in the turbidity, representing the aggregation of CS, was measured after incubation with various concentrations of recombinant proteins at 45°C for 80 min. Values are expressed as per cents of the absorbance of CS in the absence of additional proteins (100%). (c) C- and (d) N-terminal truncation series. BSA, bovine serum albumin. Experiments (c and d) were repeated three times and essentially identical results were obtained. The data of one typical experiment are represented.

Fig. 3. Effects of amino acid substitutions in the hydrophobic segment. (a) The amino acid sequences around the hydrophobic segment of 4 HSP90-family proteins are compared. Arrowheads indicate Leu-Leu replaced to Ser-Ser at amino acids 665 and 666 or at 671 and 672. Asterisks indicate identical amino acids. A bar represents the

hydrophobic region (amino acids Leu662-Leu678 of human HSP90 α). (b) SDS-PAGE of GST-HSP90 α 657-732 (lane 1), GST-HSP90 α 657-732 L665S/L666S (lane 2), GST-HSP90 α 657-732 L671S/L672S (lane 3) and GST (lane 4). M, low-molecular-weight markers. (c) The increase in the turbidity of CS (8 μ g) with increasing amounts of recombinant proteins was measured as described in “Experimental Procedures”. Experiments were repeated three times and identical results were obtained. The data of one typical experiment are represented.

Fig. 4. Suppression of the heat-induced aggregation of CS by the C-terminal domain of GRP94. (a) One microgram of GST-GRP94-M (lane 1), GST-GRP94-C (lane 2) and GST (lane 3) were electrophoresed on SDS-PAGE. M, low-molecular-weight markers (b) The increase in the turbidity of CS (8 μ g) with increasing amounts of GST-GRP94-M and GST-GRP94-C was measured. Experiments were repeated twice and identical results were obtained. The data of one typical experiment are represented.

Fig. 5. Hydropathy plot of the C-terminal domain of the 3 HSP90-family proteins. The hydropathy of the C-terminal domain of human HSP90 α (light line), human GRP94 (dotted line) and *E. coli* HtpG (bold line) were plotted according to the methods of Kyte and Doolittle [41]. The amino acid numbers are represented as those of human HSP90 α .

Table 1. Effect of amino acid substitutions in the hydrophobic segment in the C-terminal domain of HSP90 α . The bacterial 2-hybrid system was used to evaluate the binding activity. The binding activity of the C-terminal domain (100%) or its mutated forms toward the middle-C-terminal domains was quantified as the β -galactosidase activity of the bacterial 2-hybrid system.

pKT25 ^{kan} -	pUT18C ^{amp} -	Activity (%) ¹
vector	vector	6.5 \pm 0.6
HSP90 α -C	HSP90 α -MC	100.0 \pm 0.8
HSP90 α -C L665S/L666S	HSP90 α -MC	9.5 \pm 0.4
HSP90 α -C L671S/L672S	HSP90 α -MC	8.6 \pm 2.4

¹mean \pm S.D. (n=4).

Table 2. Interaction between the middle and C-terminal domains of GRP94.

The bacterial 2-hybrid system was used to evaluate the binding activity. The value of the combination of the middle and the C-terminal domains was set to 100%.

pKT25 ^{kan} -	pUT18C ^{amp} -	Activity (%) ¹
vector	vector	2.1 ± 0.4
GRP94-M	GRP94-C	100.0 ± 0.7
GRP94-M	GRP94-M	1.8 ± 0.5
GRP94-C	GRP94-C	2.5 ± 0.2

¹mean ± S.D. (n=3).

Table 3. Hybrid dimer formation in the C-terminal regions of 3 HSP90-family proteins. The bacterial 2-hybrid system was used to evaluate the binding activity. The value of the combination of pKT25^{kan}-HSP90 α -MC and pUT18C^{amp}-HSP90 α -MC was set to 100%.

pKT25 ^{kan} -	pUT18C ^{amp} -	Activity (%) ¹
vector	vector	10.1 \pm 0.2
HSP90 α -MC	HSP90 α -MC	100.0 \pm 1.3
GRP94-MC	HSP90 α -MC	13.9 \pm 6.9
GRP94-MC	HtpG-MC	81.2 \pm 40.7
HSP90 α -MC	HtpG-MC	87.7 \pm 38.6

¹mean \pm S.D. (n=4)

Fig. 1

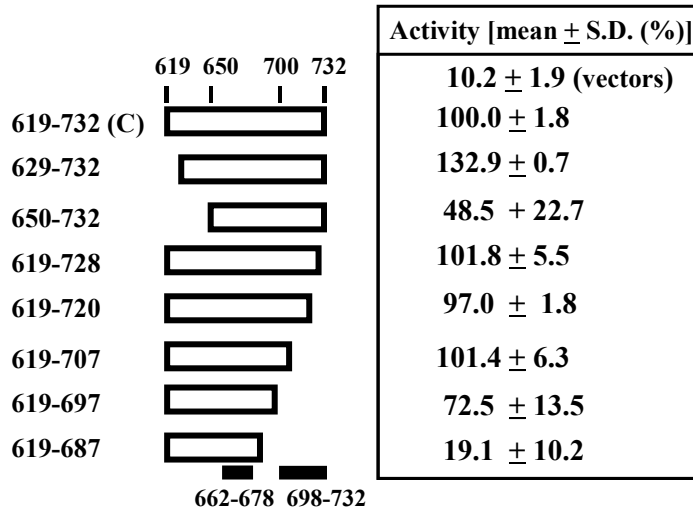
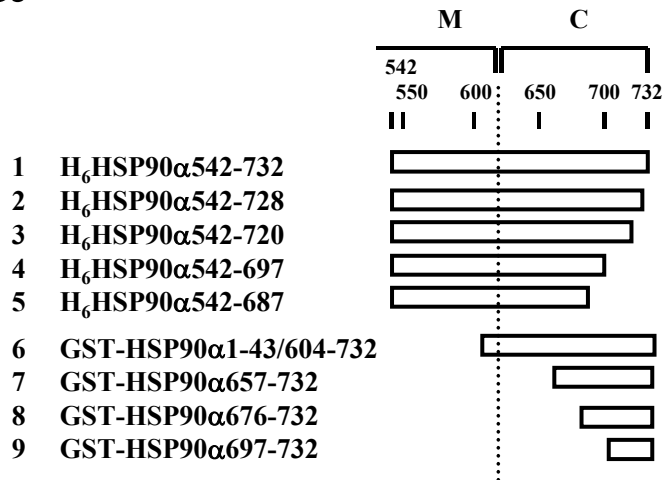
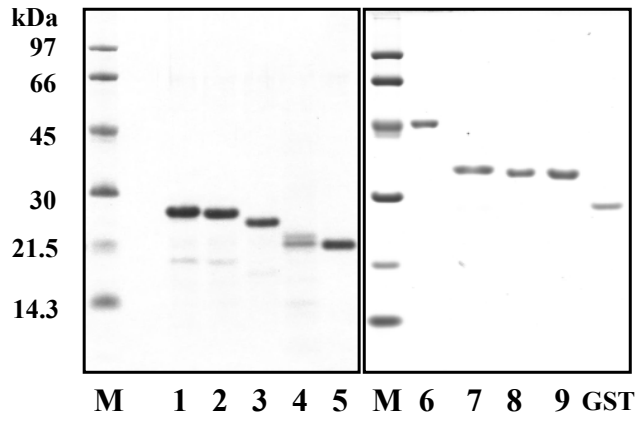


Fig. 2

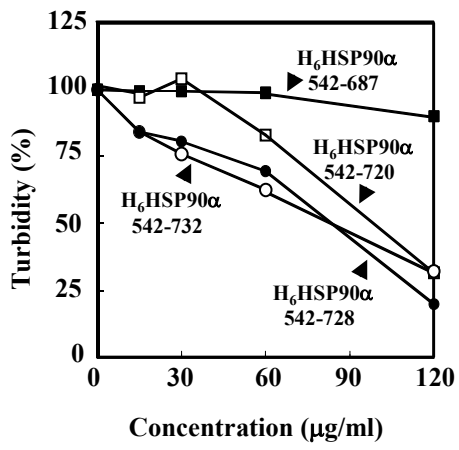
a



b



c



d

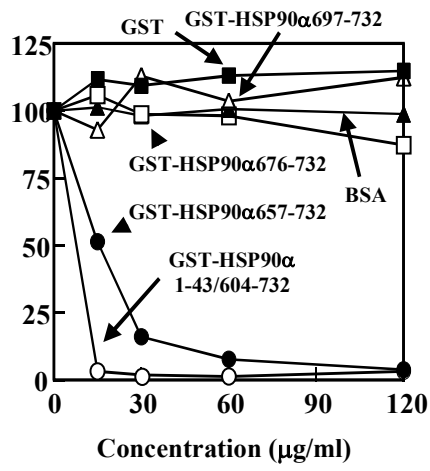
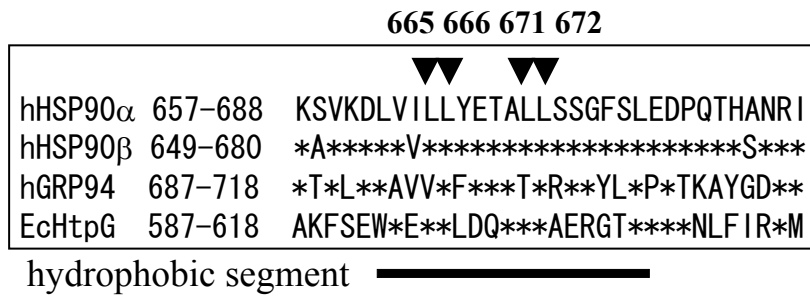
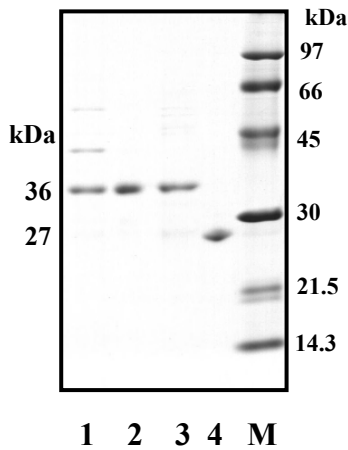


Fig. 3

a



b



c

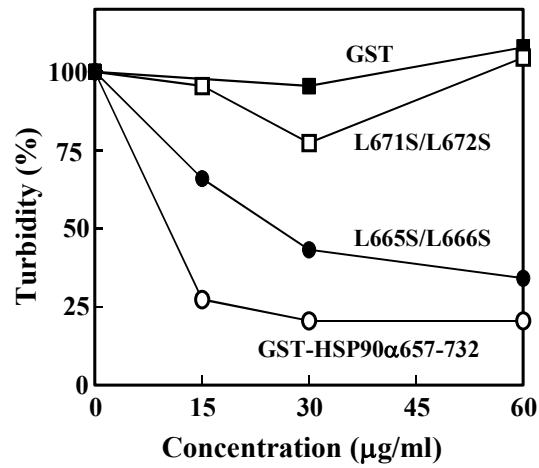


Fig. 4

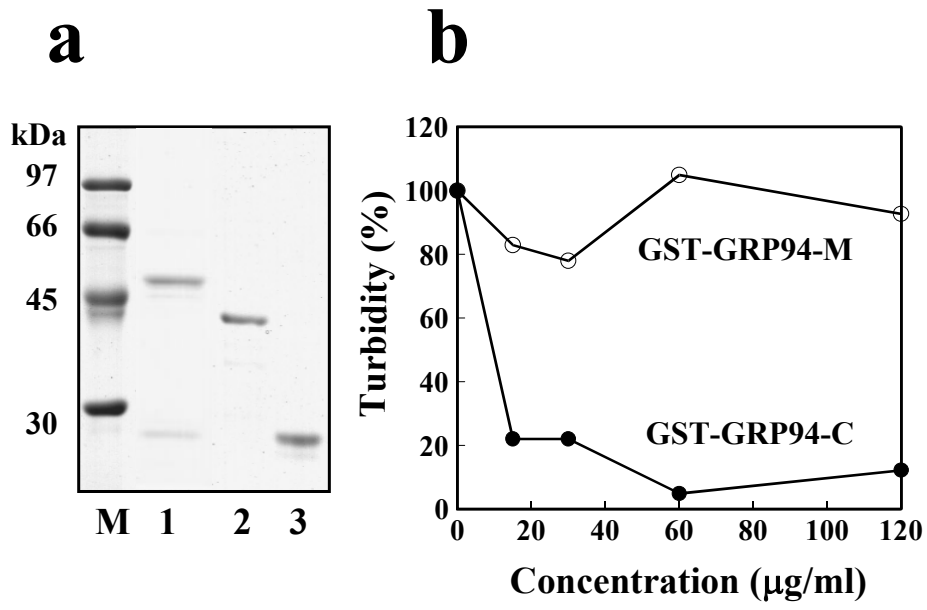


Fig. 5

