Interaction between the N-terminal and Middle Regions Is Essential for the in Vivo Function of HSP90 Molecular Chaperone*

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At the primary structure level, the 90-kDa heat shock protein (HSP90) is composed of three regions: the N-terminal (Met1–Arg400), middle (Glu401–Lys615), and C-terminal (Asp621–Asp732) regions. In the present study, we investigated potential subregion structures of these three regions and their roles. Limited proteolysis revealed that the N-terminal region could be split into two fragments carrying residues Met1 to Lys281 (or Lys283) and Glu282 (or Tyr284) to Arg400. The former is known to carry the ATP-binding domain. The fragments carrying the N-terminal two-thirds (Glu401–Lys546) and C-terminal one-third of the middle region were sufficient for the interactions with the N- and C-terminal regions, respectively. Yeast HSC82 that carried point mutations in the middle region causing deficient binding to the N-terminal region could not support the growth of HSP82-depleted cells at an elevated temperature. Taken together, our data show that the N-terminal and middle regions of the HSP90 family protein are structurally divided into two respective subregions. Moreover, the interaction between the N-terminal and middle regions is essential for the in vivo function of HSP90 in yeast.

The 90-kDa heat shock protein (HSP90)1 has been demonstrated to be an important molecule, chaperoning a variety of cellular proteins, such as steroid receptors (1–3), protein kinases involved in signal transduction (4–6), and even retrovirus reverse transcriptase (7) and endothelial nitric-oxide synthase (8) (for reviews, see Refs. 9 and 10). HSP90 occupies a central part of the chaperone network, the “foldsome,” and functions in cooperation with other chaperones and co-chaperones, such as immunophilins, CDC37/p50, HSP70, p23, Hip, Hop/p60, and PA28 (11–13) (for reviews, see Refs. 9 and 10).

This assembly process of the HSP90-substrate protein complex requires ATP (14, 15), which induces a conformational change in HSP90 (16–18). Recently, it was demonstrated that HSP90 is capable of linking substrates for degradation by the ubiquitin-proteasome pathway by cooperating with the E3 ligase carboxyl terminus of HSC70-interacting protein (CHIP) (19–21). Thus, HSP90 may play a central role in deciding the fate of proteins, refolding or degradation.

HSP90 family proteins are composed of three regions at the primary structure level (22, 23). In the present study using human HSP90α, we denote the N-terminal region, Met1–Arg400, as Region A; the middle region, Glu401–Lys615, as Region B; and the C-terminal region, Asp621–Asp732, as Region C (23). The N-terminal domain (residues 9–232) defined as the ATP/geldanamycin-binding region (24, 25), the tertiary structure of which has been clarified (24–26), corresponds to the N-terminal half of Region A. Promoting the ATP-dependent dimerization of the N-terminal domain independent of the C-terminal dimeric region (27).

Region B and Region C mediate dimerization of the HSP90 family proteins; Region B of one subunit is associated with Region C of another subunit in an antiparallel fashion (22). Electron microscopy showed that an HSP90 dimer consists of four linearly arranged globules (18), and the N- and C-terminal immunogenic sites (23) were localized in the terminal and interior globules, respectively (28).

To accomplish the molecular function of HSP90, each region may have additional roles that should be unveiled. For instance, although the ATP binding site has been localized toward the amino terminus of HSP90, ATP binding as well as elevated temperature bring about a profound conformational change that is not restricted to the ATP-binding domain (16–18). When the concentration of HSP90 was lower than 1 μM, both ATP binding and elevated temperature induced an equivalent conformational change, converting HSP90 from a linear dimer into an O-ring-shaped structure (18). On the other hand, when the concentration of HSP90 was sufficiently high, HSP90 self-oligomerized instead of formed O-ring-shaped molecules, probably through essentially identical interactions (29). Alteration of the regional interaction may be closely related to these conformational changes. In this connection, we recently proposed that the liberation of the N-terminal client-binding region from the middle suppressor region is the mechanism underlying the temperature-dependent activation of HtpG, an

Escherichia coli homologue of mammalian HSP90 (30).

Our previous study on limited proteolysis of human HSP90α strongly suggested the existence of subregional structures within the three respective regions (23). Further structural and functional analyses on potential substructures presented difficulty because of limited information on regional functions in those days. We recently reported the regional structures and their interactions of HtpG (28). As a result, several characteristics of HtpG and HSP90 regions emerged, which provided the probes for investigation of the subregional structures of HSP90.

In the present study, we investigated potential subregional structures in the three regions of HSP90 responsible for the regional interactions and further investigated the role of the regional interactions of HSP90 in vivo by using budding yeast, Saccharomyces cerevisiae. We demonstrate that the intramolecular interaction between Regions A and B is indispensable for the in vivo function of HSP90.

EXPERIMENTAL PROCEDURES

Materials—Expression vector pQE9 and plasmid pREP4 were purchased from Qiagen Inc., Chatsworth, CA. A low-sodium gel blotting salt and peptide markers were from Amersham Biosciences AB (Uppsala, Sweden). Restriction enzymes and DNA-modifying enzymes were from Nippon Gene (Tokyo, Japan). Talon metal affinity resin was obtained from CLONTECH Laboratories Inc. (Palo Alto, CA). Trypsin (5,200 USP units/mg of protein), chymotrypsin (11000 Ac-Tyr-OEt-hydrolyzing units/mg of protein) and N-acetyl-L-lysyl-L-phenylalanine chloromethyl ketone were purchased from Sigma. Yeast protein extraction buffers (36). The DNA fragments encoding Region A of HSP90 were digested with XmnI and EcoRI and ligated into the EcoRI site of expression vector pH6HSP90, which contained pKT25kan and pUT18Camp were generously provided by Drs. D. Ladant (Pasteur Institute, Paris, France) and L. Selig (Hybrigenics, S.A., Paris, France). All other reagents were of analytical grade.

Construction of Bacterial Expression Vectors—The DNA encoding the full-length form of human HSP90α (31) and E. coli HtpG (32) were generously provided by Drs. K. Yokoyama (Riken Life Science Center, Tsukuba, Japan) and E. A. Craig (University of Wisconsin Medical School, Madison, WI), respectively. Construction of the plasmids carrying the 5′-non-coding region of the intact form. The 15-kDa fragment had two adjacent N-terminal sequences of proteolytic fragments, separated proteins in a polyacrylamide gel were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). After having been stained with Coomassie Brilliant Blue, the excised bands were directly subjected to sequencing with a model 477A protein sequencer (PE Biosystems).

Expression and Purification of Recombinant Proteins—H90α, H1-HtpG, and their truncated forms were expressed and purified by use of Talon affinity resin according to the manufacturer’s protocol, except that 10 mM imidazole was added in the lysis/washing buffer. Bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol. Substitution of Conserved Amino Acids in Region B—Three amino acids within Region B were substituted with Ala in human HSP90α, E. coli HtpG, and yeast HSC82 by site-directed mutagenesis in combination with the DpnI degradation elimination of template DNA. The mutations were confirmed by DNA sequence analysis. Leu77, Glu77, and Leu92 of human HSP90α are equivalent to the respective Leu416, Glu416, and Leu422 in E. coli HtpG (34) and to Leu422, Glu422, and Leu427 in yeast HSC82 (35). Throughout the present study, we use the amino acid numbers of human HSP90α to refer to these three amino acids for simplicity.

Bacterial Two-hybrid System—Minimal regions responsible for the interaction between Region A and Region B were determined by the bacterial two-hybrid system according the method of Karimova et al. (36). The DNA fragments encoding Region A of HSP90α and its truncated forms amplified by PCR were inserted into pKT25 and designated pKT25-HSP90αA (1–400), pKT25-HSP90αB (222–400), and so on (see Fig. 3a). The DNA fragments carrying Region B of HSP90α and its truncated regions were amplified by PCR and inserted into pUT18Camp designated pUT18C-HSP90αB (601–401–600), pUT18C-HSP90αC (601–401–600), and so on (see Fig. 3b). The complex formation between co-expressed recombinant proteins was quantified by β-galactosidase activity as described previously (36). Values were reported as means of three or four samples and expressed as percentages of that activity in the bacteria co-expressing nontruncated Region A and Region B (100%). Yeast Expression System—Temperature sensitivity of yeast cells expressing mutated forms of yeast HSC82 was examined as described previously (37). 5C2HIS (MATa ura3-52 lys2-801 rur1 ade2-101 tyr1 thr5 ade6-101800 triple-Δ63 his3-Δ200 leu2-1Δ his2-HIS3 his3-HSP82 LEU2) is a strain whose endogenous HSC82 gene was disrupted and HSP82 gene was controlled with a GALI promoter (37). The strain was transformed on SGA plates by the expression of HSP82 but not on S.D. plates. A DNA fragment encoding the full-length form of yeast HSC82 was cut out with SfiI and blunt-ended was inserted into a blunt-ended BamHI site of the GPD promoter of a multicopy plasmid, pYO326GPD (38), designated pYO326GPD-HSC82. The colony formation was examined on S.D. plates at 25 or 37 °C for 3 days and 14 °C for 2 weeks following the introduction of pYO326GPD-HSC82. Transformants grew on both S.D. and SGA plates at 14–37 °C.

SDS-PAGE and Protein Sequencing—Electrophoresis was performed at a polyacrylamide concentration of 12.5% in the presence of 0.1% SDS. In cases (see Figs. 1 and 2) where fine separation of proteins smaller than 20 kDa was required, the Tris-Tricine system at a polyacrylamide concentration of 10% was employed (39). Separated proteins were stained with Coomassie Brilliant Blue. Low molecular weight and peptide markers were used as references. For determination of the N-terminal sequences of protolytic fragments, separated proteins in a polyacrylamide gel were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). Immunoblotting Analysis—Yeast cells (pYO326GPD-HSC82 and pYO326GPD-HSC82-LYA7A/H517A) cultured in SD medium overnight at 30 °C were diluted to 0.15 in absorbance at 600 nm in 50 ml of the same medium and then further cultured at 30 or 37 °C for 6 h. The cells were lysed with Y-Per at 2.5 ml/mg cell precipitate. SDS-PAGE and immunoblotting were performed thereafter as described previously (23). An anti-HSP90 monoclonal antibody, K41220 (10 μg/ml), was used as the first antibody. K41220 binds to human HSP90α and HSP90β with equal efficiency (23). It recognizes one of the most immunogenic regions of human HSP90α, 33NKTPKWPTRNPDD134 (34), of which two amino acids (underlined) are replaced in human HSP90β (33NKTPKWPTRNPDD134) (40) and yeast HSC2 (33NKTPKWPTRNPDD134) (35) as described previously. Polyclonal antibodies against a conformational epitope (triatetracontapeptide-goat anti-mouse IgG was used as the second antibody at a 1:2500 dilution. K aleidoscope prestained standard (Bio-Rad) was used as molecular markers.

RESULTS

Limited Proteolysis of Region A—We first performed limited proteolytic analysis of HSP90α. To make the interpretation simple, we here used the regions of HSP90α instead of the full-length form. Limited proteolysis of Region A with trypsin produced a limited number of proteolytic fragments (Fig. 1a). At the lowest trypsin concentration, the 52-kDa Region A was split into 15- and 40-kDa fragments (lanes 2–4). At moderate and higher trypsin concentrations, the respective 30-kDa (lanes 4–7) and 10-kDa (lanes 6–8) fragments appeared. The 40- and 10-kDa fragments carried the N terminus identical to that of the intact form. The 15-kDa fragment carrying an adjacent N-terminal form at Glu282 and Tyr284 (Table 1, lanes 2–4) and 40-kDa fragments were also produced by limited proteolysis of the full-length HSP90α (23), which implies that the proteolytic pattern is identical between the full-length form and Region A. Interestingly, the 40-kDa fragment was still blotted by an anti-HSP90 monoclonal antibody K41102, which recognizes residues 247–257, but the 30-kDa fragment was not blotted by
FIG. 1. Limited proteolysis of Region A. a, histidine-tagged Region A of HSP90α (HSP90αA, 50 μg/25 μl) was incubated for 6 h at 30 °C without (lane 1) or with 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μg (lanes 2–8) of trypsin that had been treated with N-acetyl-l-tyrosyl-l-phenylalanine chloromethyl ketone. Then aliquots (5 μg) were subjected to SDS-PAGE using the Tris-Tricine system. Apparent molecular masses of major fragments are indicated on the left, M; low molecular weight and peptide markers. The remainders of the digests were separated as above, and then the separated fragments were subjected to N-terminal sequencing. b, trypic peptides are aligned according to their N-terminal sequences. The arrows indicate tryptic cleavage sites.

| Fragment | Detected amino acids | Deduced sequence |
|----------|----------------------|------------------|
| 40       | MGSHHHHHHH            | −12MGSHHHHHH−3   |
| 30a*     | RGSHHHHHHH            | −12rgshhhhh−3    |
| 30b*     | RGS                  | −12rgshhh−4      |
| 15a*     | YIDQQEELNK            | 285K             |
| 15b*     | EKYEIDQQEEL           | 285K             |
| 10       | GSHHHHHS             | −10gshhhhhs−1    |

* Mixture of two peptides.

TABLE 1

N-terminal sequences of tryptic fragments of H6/HSP90αA

Amino acids in lowercase letters are derived from the N-terminal tag peptide. The arrows indicate tryptic cleavage sites.

Region B was successively degraded at the N-terminal side into 24-, 20-, and 19-kDa fragments. In addition to the N-terminal truncation, an 8-kDa fragment was obtained by cleavage at the C-terminal side of 27-kDa Region B. Taken together, the 42-kDa Regions BC was initially split into the 27-kDa Region B and the 14-kDa Region C, and the former was further processed into smaller fragments at cleavages of Lys546–Asn415, Lys446–Leu447, Lys458–Leu459, or Lys546–Glu547 (Fig. 2b). It should be noted that a 22-kDa fragment carrying the N-terminal two-thirds of Region B was observed upon limited proteolysis of the full-length form (23) but that such a species was not detected on proteolysis of Region B.

Minimal Regions Sufficient for the Interaction between Region A and Region B—Next we determined the minimal regions required for the interaction between Region A and Region B by using a bacterial two-hybrid system. As shown in Fig. 3a, an interaction between Region A and Region B was ascertained. This interaction reflects the intramolecular interaction of an intact molecule, as reported recently (28). HSP90α-(289–400) and HSP90α-(289–400) (i.e. truncations from the N terminus to Phe221 and to Glu288, respectively) still possessed considerable binding activities. A further deletion up to Gly310 resulted in a loss of the binding. In contrast, even the smallest deletion (28 residues) from the C terminus of Region A caused complete loss of the activity. Thus, the minimal region of Region A required for the binding to Regions BC was defined as residues 289–400.

We then defined the minimal region of Region B for binding to Region A (Fig. 3b). When the C terminus of Region B was serially deleted, residues 401–546 retained complete binding to Region A, but residues 401–541 (i.e. an additional 5-amino acid deletion) resulted in complete loss. It should be emphasized that one of the tryptic cleavage sites in Region B was Lys456–Glu547 (Table II). To the contrary, even a 20-amino acid deletion of the N terminus resulted in complete loss. Thus, residues 401–546 are the minimum requirement for the binding to Region A. From these two experiments, we conclude that residues 289–400 of Region A are associated with residues 401–546 of Region B.

Effect of Substitution of Conserved Amino Acids in Region B—The Region A–Region B interaction is maintained in both E. coli HtpG and human HSP90α, and Region B of one species can...
be replaced by that of the other species in vitro (30). Thus, amino acids within Region B conserved among the HSP90 family members should be important for the interaction. Within Region B of human HSP90α (34), HSP90β (40), human TRAP1 (mitochondrial form) (42), human GRP94/GRP96 (endoplasmic reticulum form) (43), E. coli HtpG (32), yeast HSC82 (35), and HSP82 (44), there are 28 identical amino acids, of which 25 and 3 residues are distributed in the residue sequences 401–546 and 547–615, respectively. Therefore, we expressed Regions BC with amino acid substitutions and tested their bindings to Region A; two of them (Leu477 and Glu517) were arbitrarily chosen from the N-terminal subsite, and one (Leu592) was from the C-terminal one.

The two-hybrid system revealed that the amino acid substitution in Region B of human HSP90α (Leu477 or Glu517 to Ala) caused this region to lose its ability to bind Region A, whereas the substitution at position 592 from Leu to Ala still allowed their bindings to Region A, whereas Leu592, located in the C-terminal subsite (residues 547–615), was not crucial. We also performed the experiment in the hybrid combination of these mutated HSP90αBC and HtpGA, substituting HSP90αA, and obtained an identical binding profile (Table III).

Although the two-hybrid analysis provided convincing results with positive data, there might be several interpretations on negative ones. For instance, steric hindrance caused by the fusion with a protein encoded by a vector might prevent the interaction of a protein of interest. Therefore, we further examined the effect of the mutations on the regional interaction

![fig2](image)

FIG. 2. Limited proteolysis of Regions BC. a, histidine-tagged Regions BC of HSP90α (HSP90αBC; 50 μg/25 μl) was incubated for 6 h at 30 °C without (lane 1) or with 0.002, 0.006, 0.02, 0.06, 0.2, 0.6, and 2 μg (lanes 2–8) of trypsin that had been treated with Nα-acetyl-L-tosyl-L-phenylalanne chloromethyl ketone. Thereafter, aliquots (5 μg) were subjected to SDS-PAGE using the Tris-Tricine system. Apparent molecular masses of major fragments are indicated on the left. M, molecular markers. N-terminal sequencing of the fragments were performed as in Fig. 1a. b, tryptic peptides are aligned according to their N-terminal sequences and apparent molecular masses. The arrows indicate tryptic cleavage sites.
The effect on point mutations within Region B of HSP90α was determined in terms of the interaction with Region A of HSP90α and HtpG. The β-galactosidase activities to respective intact forms (wt) were set to 100%.

| pKT25sim  | pUT18comp  | Activitya |
|-----------|------------|-----------|
| Control   | Control    | 5.8 ± 1.2 |
| Hsp90α    | Hsp90αBC wt| 100.0 ± 36.6 |
| Hsp90α    | Hsp90αBC L477A | 6.0 ± 1.1 |
| Hsp90α    | Hsp90αBC E517A | 7.7 ± 2.6 |
| Hsp90α    | Hsp90αBC L592A | 29.2 ± 8.8 |
| Control   | Control    | 15.8 ± 1.8 |
| HtpGA     | Hsp90α wt  | 100.0 ± 27.0 |
| HtpGA     | Hsp90αBC L477A | 17.0 ± 2.8 |
| HtpGA     | Hsp90αBC E517A | 13.5 ± 2.0 |
| HtpGA     | Hsp90αBC E517A | 45.2 ± 24.4 |

a Mean ± S.D. (n = 3).

by use of purified regions in vitro. Because the regions of human HSP90α readily self-oligomerize even under nonstress conditions (30), we here expressed the regions of HtpG as substitutions that carried mutations at equivalent positions. Purified preparations of Region B (H6HtpGB) of HtpG and its mutated forms as well as those of Region A (H6HtpGA) migrated as a single band on SDS-PAGE (Fig. 4a) and on PAGE under nondenaturating conditions (30), we here expressed the regions of HtpG as substitutions that carried mutations at equivalent positions. Purified preparations of Region B (H6HtpGB) of HtpG and its mutated forms as well as those of Region A (H6HtpGA) migrated as a single band on SDS-PAGE (Fig. 4a) and on PAGE under nondenaturating conditions (30). The upper shift of H6HtpGB-E517A might have been caused by the reduction in the acidity (Fig. 4b, lane 4). A retarded band appeared after mixing of H6HtpGA and H6HtpGB (lane 8). After mixing of H6HtpGBC-L477A or H6HtpGBC-E517A with H6HtpGA, retarded bands (lanes 9 and 10) still appeared and were indistinguishable from the H6HtpGA-intact H6HtpGBC complex (lane 8). Then we expressed H6HtpGBC with double mutations (i.e., H6HtpGBC-L477A/E517A). Retardation was scarcely detected, and most of the recombinant proteins migrated to the unaltered positions (lane 11). This phenomenon was specific for H6HtpGBC-L477A/E517A, because double mutations of alternate pairs still possessed considerable binding abilities (lanes 12 and 13).

We recently reported the temperature-dependent dissociation of the interaction between Region A and Region B of HtpG, which appears to be closely related to the function of the molecular chaperone (30). Accordingly, we further examined the effect of temperatures on the interaction between H6HtpGA and mutated H6HtpGBC. Essentially all H6HtpGBC associated with H6HtpGA at 4–37 °C (Fig. 4c, lane 4, arrows). In contrast, the complex formation between H6HtpGA and H6HtpGBC-L477A/E517A was drastically reduced at all temperatures tested (lane 5, arrows). Nevertheless, it should be noted that some extents of the complex formation were consistently observed at 4 and 20 °C. The complex was still observed at 30 °C but was scarcely present at 37 °C. Thus, we conclude that a single substitution at either Leu577 or Glu547 to Ala was not sufficient for the complete disruption of the intramolecular interaction when the two regions were feasibly accessible but that the simultaneous replacement of the two amino acids could completely abrogate the interaction, in particular at 37 °C.

To investigate the conformational changes of H6HtpGBC induced by the amino acid substitutions, H6HtpGBC and its mutated forms were subjected to limited proteolysis to trypsin and papain. Fig. 4d demonstrated that H6HtpGBC-L477A/E517A was highly sensitive to trypsin. Moderate susceptibility was observed on H6HtpGBC-E517A. H6HtpGBC-L477A and an intact form were resistant to trypsin under the conditions employed. Papain treatment revealed the same order of sensitivity (data not shown). Thus, the double amino acid substitutions seemed to significantly affect the tertiary structure of Regions BC. In the final part of our study, we investigated whether these mutations would affect the in vivo function of HSP90 in a yeast expression system.

**Region A-Region B Interaction Is Important in Vivo**—Yeast 5CG2His strain, which expressed yeast HSP82 under the control of galactose-regulated promoter, formed colonies at 14–37 °C on galactose-containing but not on glucose-containing plates (37). After the introduction of pY0326GPD-HSC82, which resulted in constitutively expressed intact HSC82, the yeast strain formed colonies at 25–37 °C on glucose plates, on which expression of HSP82 was repressed. Yeast strains that expressed HSC82 with a single amino acid substitution, L477A or E517A, or with a double mutation, L477A/L592A or E517A/L592A, also formed colonies at both temperatures on glucose plates (Fig. 5, a and b). In contrast, the strain that carried HSC82 with L477A/E517A double mutations grew at 25 °C (Fig. 5a) and 30 °C (data not shown) but not at 37 °C on glucose plates (Fig. 5b). Therefore, mutated HSC82 that was deficient in the Region A-Region B interaction brought the yeast cell to high temperature sensitivity, suggesting that the interaction between Region A and Region B is essential in vivo.

Immunoblotting analysis of yeast HSC82 (Fig. 6) was performed with an anti-HSP90 monocalonal antibody K41220. After cultivation either at 30 or 37 °C for 6 h, an 82-kDa band was found in all cases (i.e., yeast cells expressing intact HSC82 or HSC82-L477A/E517A at both temperatures). The intensity was more predominant at 37 °C, presumably simply reflecting elevated metabolism at higher temperatures. Therefore, the selective proteolytic break down of HSC82-L477A/E517A protein was unlikely to explain the growth inhibition of the yeast cells, but a functional defect should occur.

Taken together, the defect in the interaction between Region A and Region B H6HtpGBC-L477A/E517A especially at lower temperatures (Fig. 4c) indicate the molecular basis that produced the high temperature-sensitive mutant of the yeast. Yeast strains that carried any mutated HSC82 were not cold-sensitive (14 °C) and had no dominant negative effect (data not shown).

**DISCUSSION**

In the present study, we performed analytically limited proteolysis of Region A and Regions BC of HSP90α using bacterially expressed protein fragments to predict the subregion structures of these regions. To apply this technique, one should carefully consider whether their proteolytic profile might be altered from that of the full-length form. The tryptic pattern of Region A appeared to be identical to that of the full-length form (i.e., the major 40- and 30-kDa fragments shown in Fig. 1a were also observed in our previous analysis using the full-length HSP90α (23)).

On the other hand, the tryptic pattern of Region B seemed to be slightly different between the region fragment and the full-length form. In other words, the 27-kDa Region B produced from the full-length form was further processed to a 22-kDa species by a cleavage at the C-terminal side (20), but this entity was not formed upon proteolysis of the region because of preferential N-terminal breakdown (Table II). The present study on Regions BC demonstrated that the Lys546–Glu547 bond corresponded to this site producing the 22-kDa species (Table II). Therefore, the limited proteolytic analyses using recombinant fragments reproduced, even if but partially, the cleavage profile of the full-length form, which made it possible for us to predict the subregion boundaries.

One might raise a possibility that the proteolysis occurred in exposed surface loops within a domain. If such fragments were
formed, they would possess sticky hydrophobic patches, which would allow interaction with various fragments. However, as exemplified in Table II, the tryptic cleavage at Lys546-Glu547 in Regions BC, and, as shown in Fig. 3b, the C-terminal deletion of five amino acids from residues 401–546 to 401–541 of Region B caused a complete loss of the binding to Region A. A similar phenomenon was observed on tryptic cleavage sites (Lys281–Glu282 and Lys283–Tyr284) of Region A (Table I) and the region (residues 289–400) responsible for the binding to Region B (Fig. 3a). These findings strongly suggested the accordance of the structural and functional units on HSP90/H9251. Moreover, it should be noted the primary tryptic site within Region A was not located within the highly charged region (residues 223–283) but was the edge of it.

Region A of HSP90/H9251 was divided into two fragments, 40 and 15 kDa. Notably, cleavage sites of Region A of HSP90/H9251 and HtpG were located at close positions (Fig. 7a). From both of those structural and functional analyses, Region A of HSP90/H9251 should be divided into two subregions (i.e., residues 1–283 and 284–400). The former comprises the ATP-binding domain (residues 9–222) and highly charged region (residues 289–400) responsible for the binding to Region B and (Fig. 3a). These findings strongly suggested the accordance of the structural and functional units on HSP90a. Moreover, it should be noted the primary trypitc site within Region A was not located within the highly charged region (residues 223–283) but was the edge of it.

Region A of HSP90α was divided into two fragments, 40 and 15 kDa. Notably, cleavage sites of Region A of HSP90α and HtpG were located at close positions (Fig. 7a). From both of those structural and functional analyses, Region A of HSP90α should be divided into two subregions (i.e., residues 1–283 and 284–400). The former comprises the ATP-binding domain (residues 9–222) and highly charged region (residues 223–283) (Fig. 7). In view of its high charge density and immunogenic properties (23), the highly charged region may be exposed to the outer surface of the molecule. This region appears to be dispensable for viability and signal transduction in yeast (45) but might have a role in modulating the function of the ATP-binding site (46).

The common cleavage site (Lys546–Glu547) in both the full-

FIG. 4. Polyacrylamide gel electrophoresis of the Region A-Regions BC complex. a, 1 μg each of HtGA (lane 1) or HtGBC without (wt, lane 2) or with amino acid substitutions (lanes 3–7) as represented above the gel were run on SDS-PAGE. Numbers of substituted amino acids are expressed as those of human HSP90a. M, low weight molecular markers. b, recombinant proteins or their combined samples (1 μg/protein) as indicated above the gel were run on PAGE under nondenaturing conditions. M, molecular markers. c, recombinant proteins or their combined samples (1 μg/protein) were run on PAGE under nondenaturing conditions at the indicated temperatures. Electrophoresis was run at a low voltage (50 V) for 5–6 h to avoid the temperature increase. Lane 1, HtGA; lane 2, HtGBC; lane 3, HtGBC-L477A/E517A; lane 4, HtGA with HtGBC; lane 5, HtGA with HtGBC-L477A/E517A. M, molecular markers. d, HtGBC (wild type), HtGBC L477A, HtGBC-E517A, or HtGBC-L477A/E517A (25 μg/μl) was digested without (lane 1) or with 1 ng (lane 2) and 3 ng (lane 3) of trypsin at 30°C for 6 h as described in the legend to Fig. 1. Samples were denatured, and an aliquot (4 μg) was run on SDS-PAGE. M, low molecular weight and peptide markers.

FIG. 5. Effect of amino acid substitutions located in Region B in yeast. Aliquots of yeast culture diluted serially one-tenth were spotted on S.D. plates. The cells were incubated at 25°C (a) or 37°C (b) for 3 days. wt, wild type of HSC82.

FIG. 6. Immunoblotting analysis of yeast HSC82. Proteins extracted from yeast cells cultured at 30 or 37°C for 6 h were run on SDS-PAGE. Separated proteins (a) and yeast HSC82 (b), respectively, were visualized by Coomassie staining and immunoblotting with K41220 as described under “Experimental Procedures.” In b, marker bands (lane M) were visible because they were prestained, not by immunochemical staining.
length form and Regions BC may be located at the boundary between functional entities. In fact, this is likely, because residues 401–546 were sufficient for binding to Region A, but a further five-amino acid deletion resulted in null binding (Fig. 3b). Moreover, our previous studies demonstrated that the C-terminal portion of the region was sufficient for binding to Region C, that Val542-Ala548 of HSP90α was capable of binding to Region C (28). Therefore, it is reasonable to conclude that the C-terminal portion of Region B interacts with Region C. In Region B of HtpG, trypsin attacked a site almost identical to that of chymotrypsin, and the two sites are relatively close to the tryptic site of HSP90α (Fig. 7b) (22). Therefore, Region B can be divided into two subregions: subregion BI (residues 401–546) specific for binding to Region A and subregion BII (residues 547–615) for interaction with Region C.

We recently proposed that liberation of Region A from Region B is important for the chaperone activity of HSP90 (30). To verify our hypothesis, we substituted amino acids of subregions BI and BII conserved among HSP90 family members. As a result, the double mutant (L477A/E517A) within the subregion BI, whose product did not associate with Region A, was unable to support the growth of an HSP82-depleted yeast cell at 37 °C. Importantly, the yeast cell could grow at 14–30 °C, but not at 30 °C, which faithfully reflected the temperature dependence of the complex formation between HtpGBC-L477A/E517A and HtpGA.

Fine resolution of the region and subregion structures of HSP90 and their roles, as presented in Fig. 7c, has provided the molecular basis for systematic analyses of amino acids screened by mutational studies. The present study on a temperature-sensitive yeast strain that carries HSC82-L477A/E517A is the first example of such analyses. Although the previous studies (37, 47, 48) reported the mutations of HSP90 that caused yeast cells to be high temperature-sensitive, the underlying mechanism remains unknown. In contrast, the mechanism on the temperature sensitivity on double mutations of yeast HSC82 reported in this study seems to be readily attributed to the defect in the intramolecular interaction between subregions AI and BI (Fig. 4c). Moreover, it is reasonable to assume that potential client proteins are increased at elevated temperatures, which may additionally contribute to the growth defect of the yeast at higher temperatures. In this respect, it is interesting to investigate whether or not the four- and three-point mutations (Fig. 7c, asterisks) occurring in subregions AI and BI, respectively, reported in previous studies (37, 47, 48) actually cause the functional defect through the disruption of the regional interaction of HSP90.

In conclusion, the present study describes for the first time the mechanism on the high temperature-sensitive mutation in vivo, coupled with the in vitro functional defect (i.e. loss of the Region A-Region B interaction).

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Fig. 7. Region and subregion structures of the HSP90 family protein. a and b, amino acid sequences around major cleavage sites of Region A (a) and Region B (b) are compared between human HSP90α and E. coli HtpG. T and C, tryptic and chymotryptic cleavage site, respectively. Dots and asterisks, deleted and identical amino acids, respectively. E (20 k), an endogenous cleavage site producing a C-terminal 20-kDa fragment (23). c, three-region (A–C) and subregion (I and II) structures of HSP90α and their roles are summarized. CR, highly charged region. The arrows indicate point mutations, L477A, E517A, and L592A, introduced in this study. Mutated amino acids in yeast HSP92 (37, 47, 48) and Droso phila HSP83 (49) giving rise to altered phenotypes are mapped (asterisks) on the sequence of human HSP90α (34).
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