

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

Shigeki Matsumoto^{1, 2, 3, #}, Etsuko Tanaka^{3, #}, Takayuki K. Nemoto^{3, ||}, Toshio Ono³,
Takashi Takagi⁴, Jun Imai⁵, Yoko Kimura⁶, Ichiro Yahara⁷, Takeshi Kobayakawa³,
Takao Ayuse¹, Kumiko Oi¹ and Akio Mizuno²

¹Department of Dental Anesthesiology, ²First Department of Oral and Maxillofacial
Surgery and ³Department of Oral Biochemistry, Nagasaki University School of
Dentistry, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan; ⁴Department of Developmental
Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University,
Sendai 980-8578, Japan; ⁵Department of Molecular Oncology and ⁶Department of
Tumor Cell Biology, the Tokyo Metropolitan Institute of Medical Science, Tokyo
113-8613, Japan; ⁷Ina Institute, Medical and Biological Laboratory, Co., Ltd., 1063-103,
Ohara, Terasawaoka, Ina-City 396-0002, Japan.

Running title: Regional interaction of HSP90 and its role

[#]Equally contributed to this study.

^{||}To whom correspondence should be addressed.

Tel.: 81 95 849 7640; Fax: 81 95 849 7642; E-mail: tnemoto@net.nagasaki-u.ac.jp

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Summary

At the primary structure level, the 90-kDa heat shock protein (HSP90) is composed of 3 regions: the N-terminal (Met1-Arg400), middle (Glu401-Lys615) and C-terminal (Asp621-Asp732) regions. In the present study, we investigated potential sub-region structures of these 3 regions and their roles. Limited proteolysis revealed that the N-terminal region could be split into 2 fragments carrying residues Met1-Lys281(or Lys283) and Glu282(or Tyr284)-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 both data show that the N-terminal and middle regions of the HSP90-family protein are structurally divided into 2 respective sub-regions. Moreover, the interaction between the N-terminal and middle regions is essential for the *in vivo* function of HSP90 in yeast.

Introduction

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, and see reviews 9, 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 and see reviews 9, 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 co-operating with the E3 ligase 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 3 regions at the primary structure level (22, 23). In present study using human HSP90 α , 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. Prodromou *et al.* (25) observed a dimeric crystallographic structure of the

N-terminal ATP-binding domain of yeast HSP90. Subsequently, they showed an 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 anti-parallel fashion (22). Electron microscopy showed that an HSP90 dimer consists of 4 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 μ M, both of 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 *E. coli* homologue of mammalian HSP90 (30).

Our previous study on limited proteolysis of human HSP90 α strongly suggested the existence of sub-regional structures within the respective 3 regions (23). Further structural and functional analyses on potential sub-structures 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 us the probes for investigation of the sub-regional structures of HSP90.

In the present study, we investigated potential sub-regional structures in the 3 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 intra-molecular interaction between Region A and Region 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, U.S.A.); and low-molecular-weight and peptide markers, from Amersham Pharmacia Biotech (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, U.S.A.). Trypsin (5,200 USP units/mg protein), chymotrypsin (11000 AcTyrOEt hydrolyzing units/mg protein) and N^α-acetyl-L-tosyl-L-phenylalanine chloromethyl ketone were purchased from Sigma (St. Louis, MO, U.S.A.). Yeast protein extraction reagent Y-PER was from Pierce (Rockford, IL, U.S.A.). Bacterial strain BTH101 (F⁻, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1(Str^r)*, *hsdR2*, *mcrA1*, *mcrB1*) and plasmids pKT25^{kan} and pUT18C^{amp} 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 DNAs 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, U.S.A.), respectively. Construction of the plasmids carrying the full-length form of HSP90α and HtpG tagged with a histidine

hexamer, designated pH₆HSP90 α and pH₆HtpG, respectively, and their regions (A or BC) were described previously (28, 33). Y1090 [pREP4] was transformed with the plasmids and selected on Luria broth agar plates containing 50 μ g/ml of ampicillin and 25 μ g/ml of kanamycin.

Expression and purification of recombinant proteins - H₆HSP90 α , H₆HtpG 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 HSP90 α , *E. coli* HtpG and yeast HSC82 by site-directed mutagenesis in combination with the *Dpn*I-degradation elimination of template DNA. The mutations were confirmed by DNA sequencing. Leu477, Glu517 and Leu592 of human HSP90 α (34) are equivalent to the respective Leu416, Glu456 and Leu532 in *E. coli* HtpG (34) and to Leu453, Glu493 and Leu567 in yeast HSC82 (35). Throughout the present study, we use the amino acid numbers of human HSP90 α to refer to these 3 amino acids for simplicity.

Bacterial 2-hybrid system – Minimal regions responsible for the interaction between Region A and Region B were determined by the bacterial 2-hybrid system according the method of Karimova *et al.* (36). The DNA fragments encoding Region A of HSP90 α and its truncated forms amplified by PCR were inserted into pKT25^{kan}, designated pKT25-HSP90 α A (1-400), pKT25-HSP90 α 222-400 and so on (see Fig. 3a). The DNA fragments carrying Region B of HSP90 α and its truncated regions were amplified by PCR and inserted into pUT18C^{amp}, designated pUT18C-HSP90 α B (401-618), pUT18C-HSP90 α 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 3 or 4 samples and expressed as percents of that activity in the bacteria co-expressing non-truncated 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). 5CG2HIS (MAT α ura3-52 lys2-801^{amber} ade2-101^{ochre} triple- Δ 63 his3- Δ 200 leu2- Δ 1 hsc82::HIS3 hsp82::GAL1-HSP82::LEU2) is a strain whose endogenous HSC82 gene was disrupted and HSP82 gene was controlled with a *GAL1* promoter (37). The strain forms colonies on SGal plates by the expression of HSP82, but not on SD plates. A DNA fragment encoding the full-length form of yeast HSC82 cut out with *SfcI* and

blunt-ended was inserted into a blunt-ended *Bam*HI site of the *GPD* promoter of a multicopy plasmid, pYO326GPD (38), designated pYO326GPD-HSC82. The colony formation was examined on SD 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 SD and SGal plates at 14-37°C.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein sequencing - Electrophoresis was performed at a polyacrylamide concentration of 12.5% in the presence of 0.1% SDS. In cases (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 proteolytic fragments, separated proteins in a polyacrylamide gel were electro-transferred 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).

Immunoblotting analysis – Yeast cells (pYO326GPD-HSC82 and pYO326GPD-HSC82-L477A/E517A) 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 6h. The cells were lysed with Y-Per at 2.5 ml/g 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, N₂₉₁KTKPIWTRNPDDI₃₀₄, (34) of which 2 amino acids (underlined) are replaced in human HSP90β (N₂₈₃KTKPLWTRNPSDI₂₉₆) (40) and yeast HSC82 (N₂₆₇KTKPLWTRNPSDI₂₈₀) (35) as described previously (23). Alkaline phosphatase-conjugated goat anti-mouse IgG was used as the second antibody at a 1:2500 dilution. Kaleidoscope prestained standard (Bio-Rad) was used as molecular markers.

Polyacrylamide gel electrophoresis under nondenaturing conditions – In order to estimate molecular configurations, recombinant proteins and their mixtures were subjected to PAGE on a 7.5% polyacrylamide gel under nondenaturing conditions (41). We found that elution buffers, *i.e.* 0.1 M imidazole (pH 8) containing 10% (v/v) glycerol for H₆HtpG, did not interfere with the interaction between recombinant proteins. Accordingly, purified proteins were used without any further treatment in this study. Electrophoresis was performed at room temperature unless otherwise described.

Separated proteins were 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 references.

Protein concentration - Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.).

Figs. 1 and 2

RESULTS

Limited proteolysis of Region A - We first performed limited proteolytic analysis of HSP90 α . To make the interpretation simple, we here used the regions of HSP90 α 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- (lanes 4-7) and 10-kDa (lanes 6-8) fragments appeared. The 40- and 10-kDa fragments had an N-terminus identical to that of the intact form. The 15-kDa fragment had 2 adjacent N-termini starting at Glu282 and Tyr284 (Table I). The 30- and 40-kDa fragments were also produced by limited proteolysis of the full-length HSP90 α (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 it (23). Therefore, the 40- and 15-kDa fragments seemed to consist of amino acids 1-281/283 and 282/284-400, respectively; and the 30-kDa fragment was derived from the 40-kDa species by deletion of the highly charged region (Glu223-Lys283), as schematically illustrated in Fig. 1b.

An identical technique was applied to Region A (Met1-Arg336) of *E. coli* HtpG. A major tryptic site (Arg236-Asn237) and chymotryptic site (Trp234-Thr235) were identified (data not shown and see Fig. 7a).

Tables I and II

Limited proteolysis of Region B – Next we performed limited proteolysis on Region B. Unfortunately, Region B of HSP90 α was not quantitatively recovered from the bacterial expression system employed, presumably because the recombinant protein was unstable. Hence, we expressed Glu401-Asp732, which consisted of Region B and Region C (designated as Regions BC). The 42-kDa Regions BC was split into several fragments including 14- and 27-kDa fragments at low trypsin levels (Fig. 2a). At moderate trypsin concentrations, 6.5-, 20- and 24-kDa fragments appeared. At higher concentrations, 8- and 19-kDa fragments accumulated. Among them, the 14-kDa fragment was the most resistant to a wide range of trypsin concentrations (lanes 2-6).

N-terminal sequencing revealed that the 14-kDa fragment started at Asp621 (Table II), implying that the fragment corresponded to Region C, as expected. The N-terminal sequence of the 27-kDa fragment was identical to that of the original fragment, indicating that the 27-kDa entity represented Region B. The N-termini of the 24-, 20- and 19-kDa fragments were Asn415, Leu447 and Leu459, respectively.

Thus, the 27-kDa 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 the smaller fragments at cleavages of Lys414-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 on limited proteolysis of the full-length form (23) but that such species was not detected on proteolysis of Region B.

Fig. 3

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 Regions B by using a bacterial 2-hybrid system. As shown in Fig. 3a, an interaction between Region A and Region B was ascertained. This interaction reflects the intra-molecular interaction of an intact molecule, as reported recently (28). HSP90 α 222-400 and HSP90 α 289-400, *i.e.* truncations from 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 Lys546-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 2 experiments, we conclude that residues 289-400 of Region A are associated with residues 401-546 of Region B.

Fig. 4

Table III and Fig. 4

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 HSP90 α 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 HSP90 α (34), HSP90 β (40), human TRAP1 (mitochondrial form) (42), human GRP94/gp96 (endoplasmic reticulum form) (43), *E. coli* HtpG (32) and yeast HSC82 (35) and HSP82 (44), there are 28 identical amino acids, of which 25 and 3 residues are distributed in the sequence of residues 401-546 and 547-615, respectively. So we expressed Regions BC with amino acid substitutions and tested their bindings to Region A: two of them (Leu477 and Glu517) were arbitrary chosen from the N-terminal sub-site, and one (Leu592) from the C-terminal one.

The 2-hybrid system revealed that the amino acid substitution in Region B of human HSP90 α (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 29% of the binding (Table III). Thus, consistent with the results on the truncated forms of Region B (Fig. 3b), the conserved amino acids, Leu477 and Glu517, located in the N-terminal sub-site (residues 401-546) of the region were essential for the binding to Region A; whereas Leu592, located in the C-terminal sub-site (residues 547-615), was not crucial. We also performed the experiment in the hybrid combination of these mutated HSP90 α BC and HtpGA, substituting HSP90 α A, and obtained an identical binding profile (Table III).

Although the 2-hybrid analysis provided convincing results on 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 by use of purified regions *in vitro*. Because the regions of human HSP90 α readily self-oligomerize even under non-stress 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 nondenaturing conditions (Fig. 4b, lanes 1-7). 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 H₆HtpGBC (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 Leu477 or Glu517 to Ala was not sufficient for the complete disruption of the intra-molecular interaction when the 2 regions were feasibly accessible but that the simultaneous replacement of the 2 amino acids could completely abrogate the interaction in particular at 37°C.

In order 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. Figure 4d demonstrated that H₆HtpGBC-L477A/E517A was highly sensitive to trypsin. Moderate susceptibility was observed on H₆HtpGBC-E517A. H₆HtpGBC-L477A as well as an intact form was 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.

Fig. 5

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-, but not on glucose-containing plates (37). After the introduction of pYO326GPD-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 E517/L592, also formed colonies at both temperatures on glucose plates (Fig. 5a 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 sensitive, suggesting that the interaction between Region A-Region B is essential *in vivo*.

Immunoblotting analysis of yeast HSC82 was performed with an anti-HSP90 monoclonal antibody K41220. After cultivation either at 30 or 37°C for 6h, an 82-kDa

band was found in all cases (Fig. 6b), *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 (14°C) sensitive 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 HSP90 α using bacterially-expressed protein fragments, to predict the sub-region 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 HSP90 α (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. That is, 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 (23), but this entity was not formed on proteolysis of the region because of preferential N-terminal break down (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 sub-region 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 5 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 and (Fig. 3a). These findings strongly suggested the accordance of the structural and functional units on HSP90 α . 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 α was divided into two fragments, 40 and 15 kDa. Notably, cleavage sites of Region A of HSP90 α and HtpG were located at close positions (Fig. 7a). From both of those structural and functional analyses, Region A of HSP90 α should be divided into 2 sub-regions, *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. 7c). 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-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 5 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-Tyr627 was associated with Region C after chymotryptic proteolysis (22) and that the C-terminal one-third of HtpG (Gln481-Lys552), equivalent to residues Val542-Ala618 of HSP90 α 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 2 sites are relatively close to the tryptic site of HSP90 α (Fig. 7b and ref. 22). Therefore, Region B can be divided into two sub-regions: sub-region BI (residues 401-546) specific for binding to Region A and sub-region 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). So as to verify our hypothesis, we substituted amino acids of sub-regions BI and BII conserved among HSP90-family members. As a result, the double mutant (L477A/E517A) within the sub-region BI, whose product did not associate with Region A, was unable to support the growth of 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.

Fig. 7

Fine resolution of the region- and sub-region 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 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 intra-molecular interaction between sub-regions AII 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 4 and 3 point mutations (asterisks in Fig. 7c) occurring in sub-regions AII 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*, in coupled with the *in vitro* functional defect, *i.e.*, loss of the Region A-Region B interaction.

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FOOTNOTE

¹The following abbreviations are used: HSP90, the 90-kDa heat shock protein; HSP90 α and HSP90 β , the α and β isoforms of HSP90, respectively; HSP82 and HSC82, heat shock protein 90 and heat shock cognate protein 90, respectively, of the budding yeast, *Saccharomyces cerevisiae*; HtpG, an *E. coli* homologue of mammalian HSP90; GRP94/gp96, the 94-kDa glucose-regulated protein (endoplasmic form of HSP90); TRAP1, mitochondrial form of HSP90; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

FIGURE LEGENDS

Fig. 1. Limited proteolysis of Region A – (a) Histidine-tagged Region A of HSP90 α (HSP90 α A, 50 μ g/25 μ l) 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 μ g (lanes 2-8) of trypsin that had been treated with N $^{\alpha}$ -acetyl-L-tosyl-L-phenylalanine chloromethyl ketone. Then, aliquots (5 μ g) 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) Tryptic peptides are aligned according to their N-terminal sequences and apparent molecular masses. Hatched boxes represent an N-terminal histidine tag (M₁₂-R-G-S-H₆-G-S₁) preceding the first Met of HSP90 α . N-terminal amino acids of the fragments are shown on the boxes.

Fig. 2. Limited proteolysis of Regions BC – (a) Histidine-tagged Regions BC of HSP90 α (HSP90 α BC, 50 μ g/25 μ l) 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 μ g (lanes 2-8) of trypsin that had been treated with N $^{\alpha}$ -acetyl-L-tosyl-L-phenylalanine chloromethyl ketone. Thereafter, aliquots (5 μ g) 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. Hatched boxes represent an N-terminal histidine tag preceding Glu401. N-terminal amino acids of the fragments are shown above the bars.

Fig. 3. Minimal regions essential for the Region A-Region B interaction – (a) Region A and its truncated forms expressed in the pKT25^{kan} vector are schematically represented. Closed, hatched and dotted boxes, respectively, represent the ATP-binding site (residues 9-222), highly charged region (residues 223-283) and immunogenic site I (residues 227-310) (23). These plasmids were co-expressed with pUT18C^{amp}-HSP90αB. (b) Region B and its truncated forms expressed with pUT18C^{amp} vector are schematically represented. They were co-expressed with pKT25^{kan}-HSP90αA. The β-galactosidase activities of the bacteria co-expressing Region B and Region A (100%) or their truncated forms were determined. Values are represented as per cents ± S.D. of 3 samples. Control means a combination with plasmids, pKT25^{kan} and pUT18C^{amp}.

Fig. 4. Polyacrylamide gel electrophoresis of the Region A-Regions BC complex - (a) One microgram each of H₆HtpGA (lane 1) or H₆HtpGBC 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

HSP90 α . M, low-weight molecular markers. (b) Recombinant proteins or their combined samples (1 μ g per protein) as indicated above the gel were run on PAGE under nondenaturing conditions. M, molecular markers. (c) Recombinant proteins or their combined samples (1 μ g per protein) were run on PAGE under nondenaturing conditions at indicated temperatures. Electrophoresis was run at a low voltage (50 V) for 5-6 h to avoid the temperature increase. Lane 1, H₆HtpGA; lane 2, H₆HtpGBC; lane 3, H₆HtpGBC-L477A/E517A; lane 4, H₆HtpGA with H₆HtpGBC; and lane 5, H₆HtpGA with H₆HtpGBC L477A/E517A. M, molecular markers. (d) H₆HtpGBC (wt), H₆HtpGBC L477A, H₆HtpGBC-E517A or H₆HtpGBC-L477A/E517A (25 μ g/50 μ l) was digested without (lane 1) or with 1 (lane 2) and 3 ng (lane 3) of trypsin at 30°C for 6h as described in Fig. 1. Samples were denatured and an aliquot (4 μ g) 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 SD plates. The cells were incubated at (a) 25°C or (b) 37°C 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 6h were run on SDS-PAGE. (a) Separated proteins and (b) yeast HSC82, respectively, were visualized by Coomassie staining and immunoblotting

with K41220 as described in Experimental Procedures. In panel b, marker bands (lane M) were visible, because they were pre-stained, not by immunochemical staining.

Fig. 7. Region and sub-region structures of the HSP90-family protein – (a and b) Amino acid sequences around major cleavage sites of (a) Region A and (b) Region B are compared between human HSP90 α and *E. coli* HtpG. T and C, respectively, represent tryptic and chymotryptic cleavage sites. Dots and asterisks represent deleted and identical amino acids, respectively. E (20 kDa) represents an endogenous cleavage site producing a C-terminal 20-kDa fragment (23). (c) Three-region (A, B and C) and sub-region (I and II) structures of HSP90 α and their roles are summarized. CR, highly charged region. Arrows indicate point mutations, L477A, E517A and L592A, introduced in this study. Mutated amino acids in yeast HSP82 (37, 47, 48) and *Drosophila* HSP83 (49) giving rise to altered phenotypes are mapped (asterisks) on the sequence of human HSP90 α (34).

Table I. N-terminal sequences of tryptic fragments of H₆HSP90αA. Amino acids written in small letters are derived from the N-terminal tag peptide. Arrows indicate tryptic cleavage sites.

Fragment (kDa)	Detected amino acids	Deduced sequence
40	MRGSHHHHHH	M-12RGSHHHHHH-3
30a ¹	RGSHHHHHH	m-12 ↓ r-11gshhhhhh-3
30b ¹	RGS	m-12rgshhhhhh-4
15a ¹	YIDQEELNK	K283 ↓ Y284IDQEELNK292
15b ¹	EKYIDQEEL	K281 ↓ E282KYIDQEEL290
10	GSHHHHHHGS	r-11 ↓ g-10shhhhhhgs-1

¹mixture of two peptides.

Table II. N-terminal sequences of tryptic fragments of H₆HSP90αBC. Amino acids written in small letters are derived from the N-terminal tag peptide. Arrows indicate tryptic cleavage sites.

Fragment (kDa)	Detected amino acids	Deduced sequence
27	MRGSHHHHHH	m-12rgshhhhhh-3
24	NLVKKXLEL	K414 ↓ N415LVKKCLEL423
21	LGIXEDS	K446 ↓ L447GIHEDS453
19	LSELLRYY	K458 ↓ L459SELLRYY 466
14	DNSTMGYMAA	R620 ↓ D621NSTMGYMAA630
8	EGLELDEDEE	K546 ↓ E547GLELDEDEE556
6.5a ¹	MRGSHHHHHH	m-12rgshhhhhh-4
6.5b ¹	HIYYITGET	K489 ↓ H490IYYITGET498

¹mixture of two peptides. X, unidentified amino acid.

Table III. Effect of point mutations within Region B of HSP90 α . The effect on point mutations within Region B of HSP90 α was determined in terms of the interaction with Region A of HSP90 α and HtpG. The β -galactosidase activities to respective intact forms (wt) were set to 100%.

pKT25 ^{kan} -	pUT18C ^{amp} -	Activity (%) ¹
control	control	5.8 \pm 1.2
HSP90 α A	HSP90 α BC wt	100.0 \pm 36.6
HSP90 α A	HSP90 α BC L477A	6.0 \pm 1.1
HSP90 α A	HSP90 α BC E517A	7.7 \pm 2.6
HSP90 α A	HSP90 α BC L592A	29.2 \pm 8.8
control	control	15.8 \pm 1.8
HtpGA	HSP90 α wt	100.0 \pm 27.0
HtpGA	HSP90 α BC L477A	17.0 \pm 2.8
HtpGA	HSP90 α BC E517A	13.5 \pm 2.0
HtpGA	HSP90 α BC E517A	45.2 \pm 24.4

¹mean \pm S.D. (n=3)

FIGURES

Fig. 1

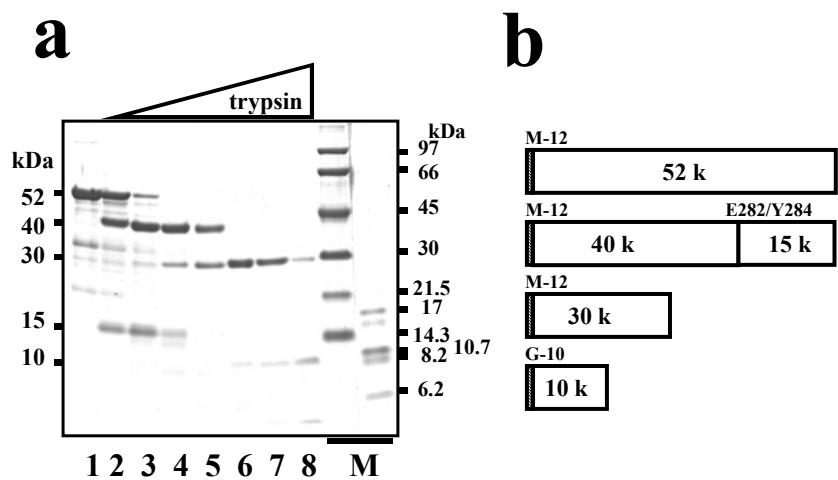


Fig. 2

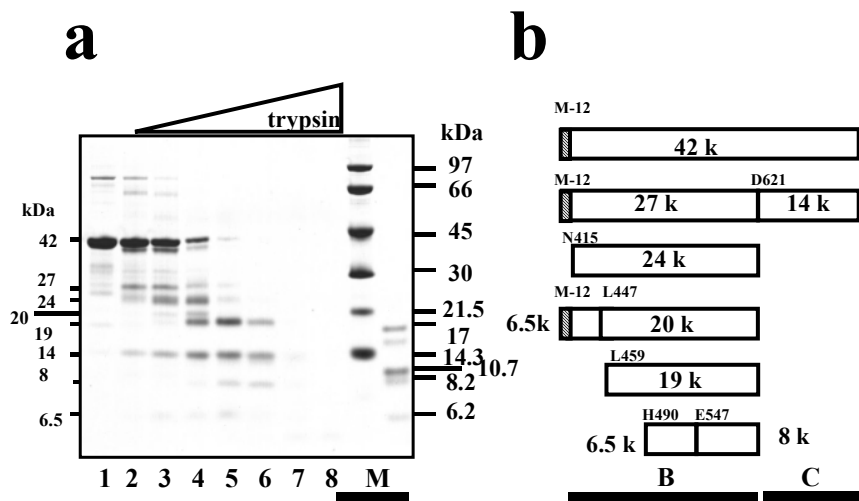


Fig. 3

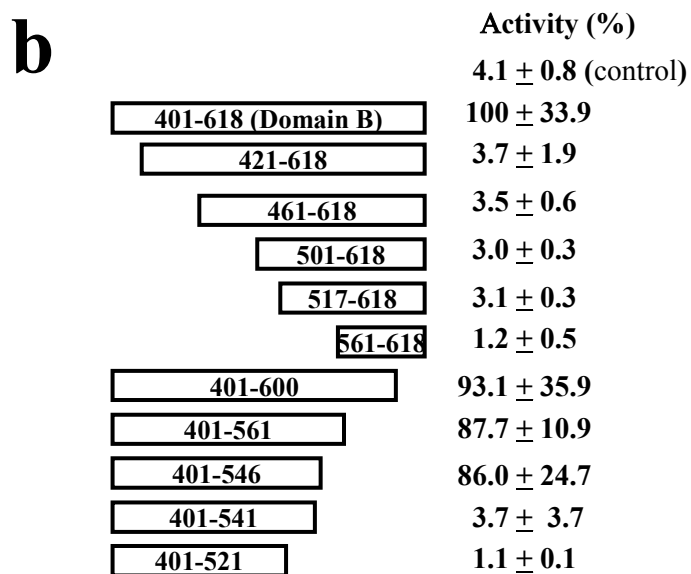
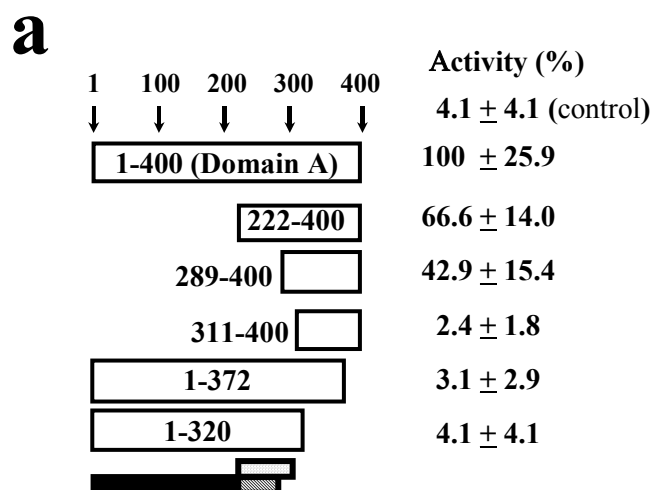


Fig. 4

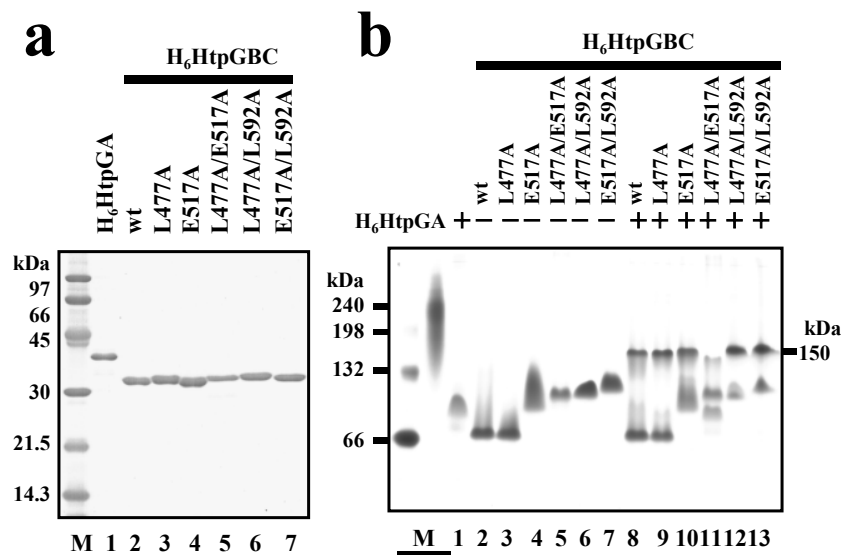


Fig. 4 (continued)

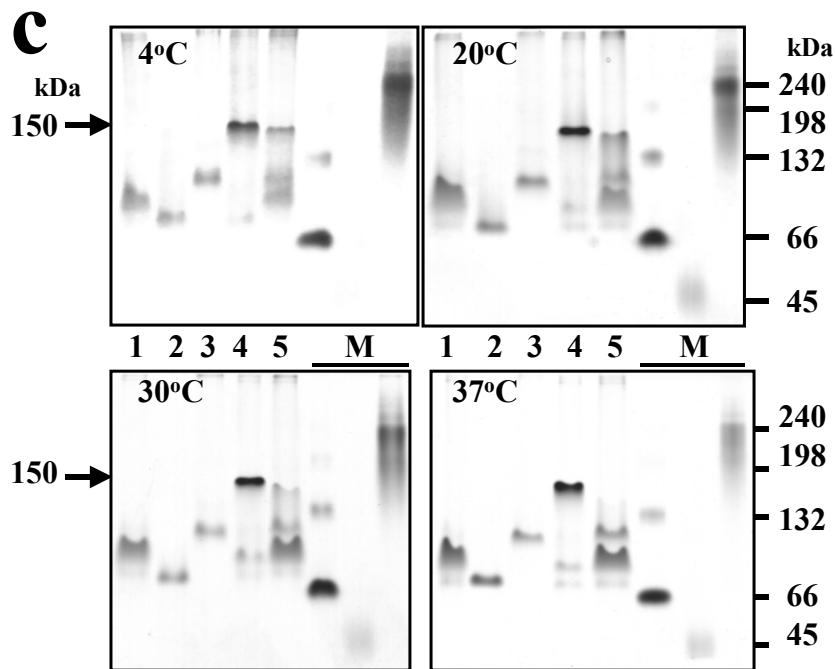


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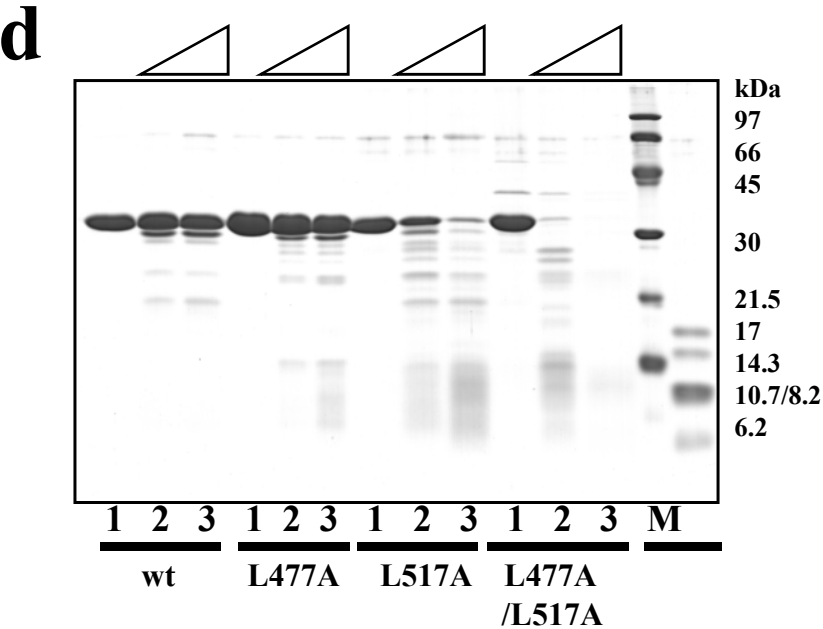


Fig. 5

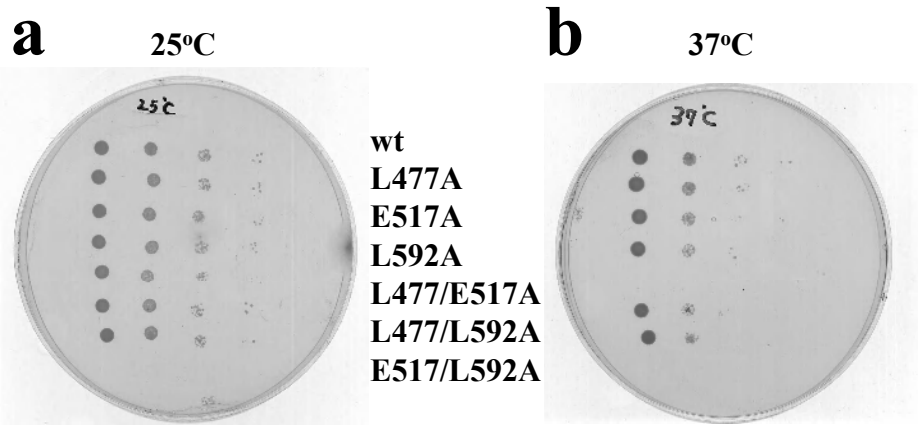


Fig. 6

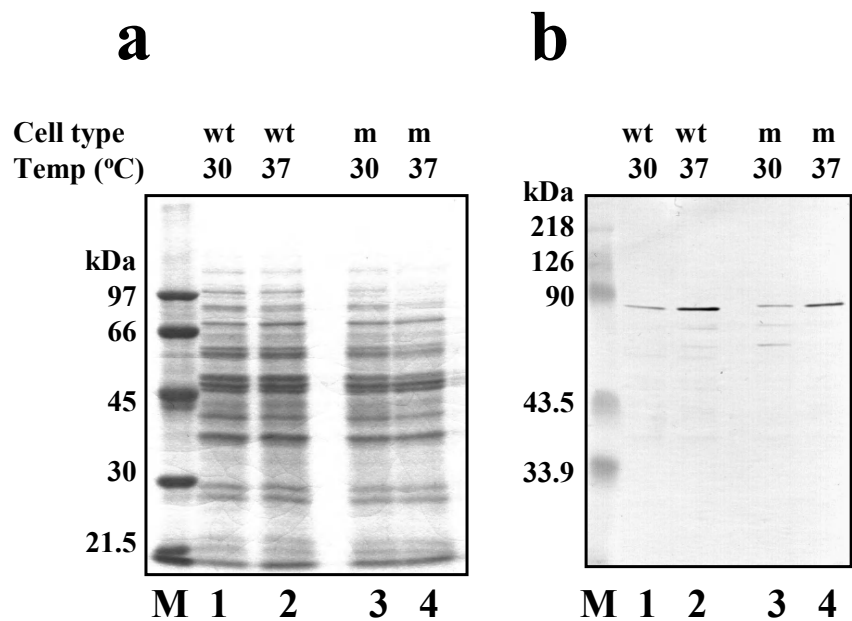


Fig. 7

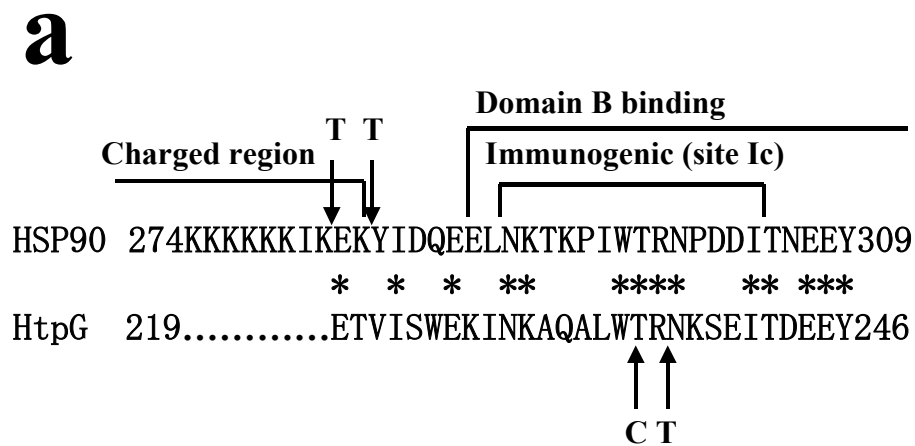


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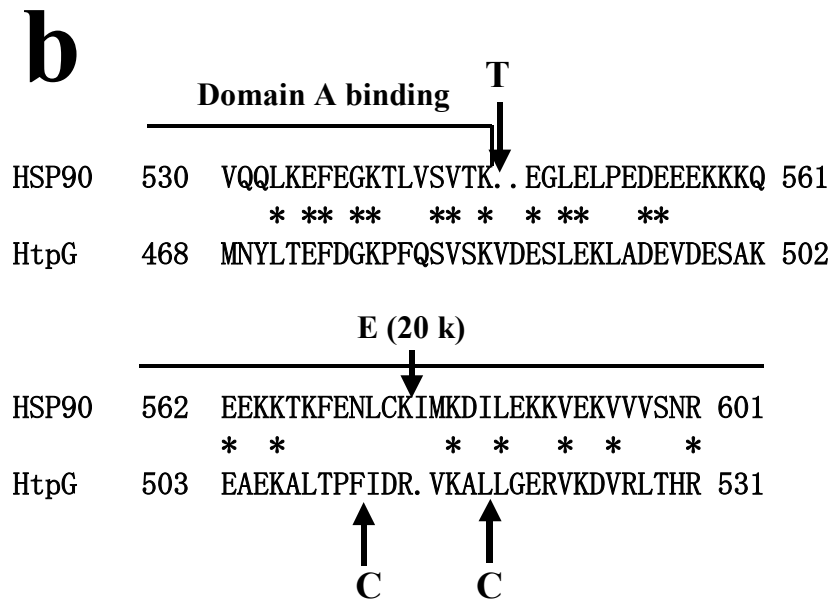


Fig. 7 (continued)

