Domain Structures and Immunogenic Regions of the 90-kDa Heat-shock Protein (HSP90)

PROBING WITH A LIBRARY OF ANTI-HSP90 MONOCLONAL ANTIBODIES AND LIMITED PROTEOLYSIS*

(Received for publication, May 30, 1997, and in revised form, July 29, 1997)

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Domain structures of the 90-kDa heat-shock protein (HSP90) have been investigated with a library of anti-HSP90 monoclonal antibodies (mAbs) and by limited proteolysis with trypsin and chymotrypsin. Thirty-three mAbs were obtained by immunization with bacterially expressed human HSP90α and HSP90β isoforms. Among them, ten and three mAbs reacted specifically with HSP90α and HSP90β, respectively. Immunoblotting and enzyme-linked immunosorbent analyses revealed that major immunogenic domains were located at two restricted regions of HSP90α, i.e. amino acids 227–310 (designated Region I) and 702–716 (Region II), corresponding to a highly charged region and a region near the C terminus, respectively. Taken together with the characteristics of the amino acid sequences, these two immunogenic regions appeared to be exposed at the outer surface of HSP90. We further investigated the domain structures of HSP90 by limited proteolysis in combination with N-terminal sequencing and immunoblotting analyses. Tryptic cleavages of HSP90α at low concentrations revealed the existence of major susceptible sites at Arg400-Glu401, Lys615-Ala616, and Arg620-Asp621. Proteolysis at higher trypsin concentrations caused successive cleavages only toward the N-terminal direction from these sites, and Region I was included in the region selectively deleted during this process, thereby further suggesting its surface location. From these results, we propose three domain structures of HSP90 consisting of amino acids 1–400, 401–615, and 621–732. Differences in the protease sensitivity and immunogenicity further suggest that every domain is composed of two subdomains. This is the first study describing the domain structures and the immunogenic regions of HSP90.

The 90-kDa heat-shock protein (HSP90)1 is one of the major stress proteins in eukaryotic cells. There are at least two HSP90 genes, and two HSP90 isoform proteins, α and β, are expressed in the cytosolic compartment (1). The amino acid sequence of HSP90α is 85 (human; see Refs. 1 and 2) to 90% (yeast; see Ref. 3) homologous to HSP90β. Either one of the isoforms is indispensable for the growth of yeast cells at higher temperatures (3). Biochemical characterization of purified HSP90 indicates that HSP90α predominantly exists as a homodimer and HSP90β exists mainly as a monomer (4). Dimer formation is mediated by the interaction at the C-terminal 191 amino acids in which the C-terminal region (Met618–Asp622) of one subunit associates with the adjacent region (Val542–Tyr557) of the other subunit (5). The amino acid substitutions at 561–685 between α and β isoforms are responsible for the impeded dimerization of HSP90β (5).

HSP90 is believed to have a chaperone-like activity for particular molecules that are involved in signal transduction, such as steroid receptors (6), casein kinase II (7), pp60cSrc (8), eIF2α kinase (9), and aryl hydrocarbon receptor (dioxin receptor) (10). HSP90 specifically binds to these proteins; and, in most cases, this interaction is essential for the function of the proteins (9, 11, 12). However, a variety of evidence, i.e. the abundance of HSP90 in cells even under nonstressed conditions, the conserved amino acid sequences from prokaryotic to eukaryotic cells (13), and the indispensability in yeast (3), strongly suggests that HSP90 is involved in more fundamental functions of cells. In fact, several studies have recently shown that HSP90 functions as a general chaperone. That is, it interacts with various proteins less specifically and modulates their conformation. For instance, the refolding of citrate synthase is significantly enhanced by the co-presence of HSP90 (14). The spontaneous refolding of denatured dihydrofolate reductase and irreversible denaturation of firefly luciferase are prevented by association with HSP90 (15). The latter study further demonstrated that the chaperone-like activity of HSP90 is closely related to the oligomerization of HSP90 at temperatures higher than 46 °C. Although HSP90 possesses an ATPase activity (16), this activity does not appear to be needed for the oligomerization of HSP90.

Epitope mapping is a useful approach for investigating the structures of proteins of interest. Several anti-HSP90 monoclonal antibodies (mAbs) have been produced to date (6, 10, 17). Although the epitopes of several anti-HSP90 mAbs have been described (18), systematic assignment of the immunogenicity of HSP90 has not been conducted. In this study, we developed a library of the mAbs that specifically recognize human HSP90 and with them demonstrated that the major immunogenic domains are located in two restricted regions. The domain structures of HSP90 were further analyzed by limited proteolysis. We finally proposed three domain structures of HSP90. In addition, this is the first report of developing isoform-specific mAbs against HSP90.

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HSP90 amino acid deletions corresponding to amino acids 1–5 and 238–240 of HSP90 isoforms were constructed by the polymerase chain reaction technique with appropriate primers as described previously (5). The details on the constructions are available on request. H6HSP90a was purified with Talon™ metal affinity resin according to the manufacturer protocol.

Preparation of Anti-HSP90 mAbs—Following thrombin cleavage of full-length forms of GST-HSP90α and β, the HSP90 moieties were purified by DEAE ion-exchange HPLC as described (20). The hybridoma cells producing anti-HSP90 mAbs were prepared according to Ochi and Herzenberg (21). The fusions were performed separately with the mice injected with HSP90α and HSP90β. Anti-HSP90 mAb-producing hybridomas were selected by use of an enzyme-linked immunosorbent assay (ELISA) with purified HSP90α or β (0.5 μg) coated on each well of a 96-well titer plate (Falcon). Peroxidase-conjugated anti-mouse (IgG plus IgM) antibodies were used as second antibodies. Absorbance at 492 nm was measured following incubation with O-phenylenediamine for 1 h at 30 °C. Positive hybridomas were cloned by limiting dilution.

Large scale production of mAbs was carried out by growing the hybridoma cells in ascites of mice. The immunoglobulin fraction was precipitated with 50% (w/v) ammonium sulfate, and the precipitate was dialyzed against saline containing 0.1% sodium azide.

Table I: Characteristics of the mAbs against human HSP90α

| No. | Name | Antigen | ELISA | Immunoblotting | Specificity | Class |
|-----|------|---------|-------|----------------|------------|-------|
|     |      |         | Reactivity to HSP90α | Reactivity to HSP90β | Reactivity to HSP90α | Reactivity to HSP90β |          |        |
| 1   | K41007 | HSP90α | 100  | 0  | ++ | – | α | IgG1 |
| 2   | K41346 | HSP90α | 100  | 0.1 | ++ | – | α | IgG1 |
| 3   | K41241 | HSP90α | 100  | 0.2 | ++ | – | α | IgG1 |
| 4   | K41230 | HSP90α | 100  | 0.3 | ++ | – | α | IgG1 |
| 5   | K41230 | HSP90α | 100  | 0.3 | ++ | – | α | IgG1 |
| 6   | K41230 | HSP90α | 100  | 0.4 | ++ | – | α | IgG1 |
| 7   | K41020 | HSP90α | 100  | 0.4 | ++ | – | α | IgG2b |
| 8   | K4116A | HSP90α | 100  | 0.6 | ++ | – | α | IgG2b |
| 9   | K41107 | HSP90α | 100  | 0.9 | ++ | – | α | IgG2b |
| 10  | K41009 | HSP90α | 100  | 1  | ++ | – | α | IgG2b |
| 11  | K41310 | HSP90α | 100  | 1.0 | ++ | – | α | IgG2b |
| 12  | K41007 | HSP90α | 100  | 1.0 | ++ | – | α | IgG2b |
| 13  | K41020 | HSP90α | 100  | 1.0 | ++ | – | α | IgG2b |
| 14  | K41016 | HSP90α | 100  | 1.0 | ++ | – | α | IgG2b |
| 15  | K41218 | HSP90α | 100  | 1.0 | ++ | – | α | IgG2b |
| 16  | K41002 | HSP90α | 100  | 9.0 | ++ | – | α | IgG2b |
| 17  | K4116C | HSP90α | 100  | 9.4 | ++ | – | α | IgG2b |
| 18  | K41220 | HSP90α | 100  | 9.7 | ++ | – | α | IgG2b |
| 19  | K41116 | HSP90α | 88.8 | 100 | ++ | + | α | IgM |
| 20  | K41338 | HSP90α | 87.2 | 100 | ++ | + | α | IgM |
| 21  | K41322 | HSP90α | 81.3 | 100 | ++ | + | α | IgM |
| 22  | K41331 | HSP90α | 75.1 | 100 | ++ | + | α | IgM |
| 23  | K41122B| HSP90α | 62.9 | 100 | ++ | + | α | IgM |
| 24  | K4125A | HSP90β | 100  | 0  | ++ | – | α | IgG2b |
| 25  | K41270 | HSP90β | 100  | 79.2 | ++ | + | α | IgG1 |
| 26  | K4127D | HSP90β | 100  | 90 | ++ | + | α | IgG1 |
| 27  | K4127D | HSP90β | 100  | 90 | ++ | + | α | IgG1 |
| 28  | K41378 | HSP90β | 53.5 | 100 | ++ | + | α | IgG1 |
| 29  | K41279 | HSP90β | 49.6 | 100 | ++ | + | α | IgG1 |
| 30  | K41316 | HSP90β | 4.5  | 100 | ++ | + | α | IgG1 |
| 31  | K41274 | HSP90β | 2.8  | 100 | ++ | + | α | IgG1 |
| 32  | K41274 | HSP90β | 0.5  | 100 | ++ | + | α | IgG1 |
| 33  | K41274 | HSP90β | 0    | 100 | –  | ++ | β | IgM |

* According to the results, isoform specificities of mAbs except K3725A are represented as follows: bold letters, HSP90α specific; not modified, HSP90α preferential; bold italic, HSP90β specific; italic, HSP90β preferential; underlined, bound to both isoforms equivalently.

* Not defined because the results of ELISA and immunoblotting were not compatible.
mg/ml) at 25 °C for 2 h. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM was used as the second antibody at a 1:5000 dilution in 50 mM Tris-HCl (pH 7.6), 0.15M NaCl (TBS) containing 0.05% Tween 20 and 0.25% bovine serum albumin. After having been washed with TBS containing 0.1% Tween 20, blots were visualized by incubation with BCIP and NBT. Low molecular weight markers and rainbow markers were used as standards for Coomassie staining and immunoblotting, respectively.

Epitope Mapping by ELISA—The regions recognized by the mAbs were investigated by ELISA and immunoblotting with various forms of HSP90s fused to GST. The purified protein (0.5 mg) coated on each well of a titer plate was incubated with 0.3 mg of purified mAbs in phosphate-buffered saline (pH 7.3). Alkaline phosphatase-conjugated goat anti-mouse IgG or anti-mouse IgM was used as the second antibody at a 1:5000 dilution. BCIP and NBT in a soluble buffer system (Kirkegaard & Perry Lab.) were used as the substrates, and absorbance at 600 nm was measured following a 1-h incubation at 25 °C.

ELISA with an Octapeptide Library—A noncleavable octapeptide library covering amino acids 212–312 of HSP90a was prepared according to the manufacturer method (Chiron Mimotopes Pty. Ltd. Clayton, Victoria, Australia). Octapeptides with their N terminus shifted sequentially to each amino acid residue from 212 to 305. The reactivity of the mAbs to the peptides was determined by ELISA as described above.

Limited Proteolysis and N-terminal Amino Acid Sequencing—Proteins in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 10% glycerol were incubated at various concentrations of trypsin (N-tosyl-L-phenylalanine chloromethyl ketone treated) or chymotrypsin (N-a-tosyl-L-lysine chloromethyl ketone treated) at 30 °C for 6 h. Proteolytic peptides (13 µg/lane) were denatured, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The protein bands visualized with Coomassie Brilliant Blue were subjected to N-terminal sequencing with a model 477A protein sequencer (Applied Biosystems) equipped with an on-line model 120A analyzer.

Phage Display System—Amino acid sequence with an affinity to an anti-HSP90 mAb (K41007) was determined by use of the phage display system with a heptapeptide library according to the manufacturer protocol. At each step of panning, 2 × 10¹¹ phages were incubated with 15 µg of the mAb precoated on a well of a 96-well Maxisorp plate (Nunc). Following four pannings, the phages were amplified and purified, and then the amino acid sequence of the heptapeptide was deduced from the nucleotide sequence.

RESULTS

Isoform Specificity and Immunoglobulin Class of the mAbs—We obtained 33 independent mAbs against human HSP90 isoforms. For determination of the isoform specificity of the mAbs, ELISA was performed with various amounts of HSP90a and HSP90b (0.07–150 ng). According to the results, we tentatively classified the mAbs into the following three classes: mAbs with cross-reactivity to the other isoform of less than 1% were isoform-specific; mAbs with the cross-reactivity to the other isoform from 1 to 66.7% were isoform-preferential; and mAbs reacting with the other isoform with more than 66.7% efficiency were equivalently recognizing ones (Table I). Unexpectedly, K3725A, obtained from a mouse immunized with HSP90b, was specific for HSP90a on the basis of ELISA.
The isof orm specificity was further investigated by immunoblotting with bacterially expressed HSP90α and HSP90β. The immunoblotting data were in accord with the isof orm specificities of the mAbs estimated by ELISA with the exception of K3725A (Table I). K3725A bound to both HSP90α and HSP90β on immunoblotting. As a result, isof orm specificities of the mAbs were assigned as shown in Table I. We obtained ten (numbers 1–10) and three (numbers 31–33) mAbs that specifically interacted with HSP90α and β, respectively; five mAbs (numbers 11–15) that preferentially interacted with HSP90α; five mAbs (numbers 23 and 27–30) that preferentially interacted with HSP90β; and nine mAbs (numbers 16–22, 25, and 26) that were equivalently reactive to both isof orms. The isof orm specificities of HSP90 were determined (Table I).

Epitope Mapping of HSP90—Next we determined the regions of HSP90 recognized by the mAbs by using various deletion mutants of HSP90α (Fig. 1a) and HSP90β. Most forms were purified to homogeneity by affinity chromatography on SDS-PAGE (data not shown). However, several forms revealed additional bands on SDS-PAGE. Even in such cases, the positions of the expressed proteins were defined by comparison with calculated molecular masses. Bands with molecular masses lower than expected seemed to be proteolytic products of the recombinant proteins. The purified samples were used for ELISA; whereas, the immunoblotting analysis was done with the bacterial lysates containing the expressed proteins to avoid proteolysis during the purification step.

Twelve forms of recombinant HSP90αα were immunoblotted with K41107, a mAb specific for HSP90α. As shown in Fig. 1b (left), specific bands and their degraded ones were observed in lanes 1, 3, and 6–8. Blots of HSP90α1–295, HSP90α121–312, and HSP90α216–312 (lanes 6–8) indicated that the epitope for K41107 resided within the sequence of amino acids 216–285. This result was further confirmed by ELISA (Fig. 1b, right, columns 6–8). K41122B, a mAb that bound to the two isof orm s equivalently, reacted with HSP90α216–312 and HSP90α1–47/290–312 (Fig. 1c, lanes 8 and 9), indicating its binding to a short region of 23 amino acids (290–312).

We performed the epitope mappings with the rest of the mAbs by the same procedures. The full-length form and four deleted forms of HSP90β were used for the investigation of the mAbs specifically or preferentially bound to HSP90β. In consequence, their recognition sites were defined as shown in Table II. These observations demonstrated that the epitopes defined by most mAbs were located in particular regions: the epitopes recognized by 25 out of the 33 mAbs (75%) were found within amino acids 185–335, most probably in 216–312. We tentatively designated this region as Region I. The second one, designated Region II, seemed to be found at the C-terminal region at 533–732. As expected, the isof orm specificity of mAbs seemed to be dependent on the degree of amino acid substitutions in the recognized region: mAbs recognizing conserved sites, such as amino acids 290–312, had no or little isof orm preference; and amino acids 216–285 containing the least conserved region (amino acids 238–273, 47% homology between HSP90α and HSP90β) were recognized by isof orm-specific or highly isof orm-preferential mAbs (Table II).

Epitope Mapping at Region I—Immunoblotting analysis and ELISA indicated specific localization of the immunogenic region (Region I) most probably within amino acids 216–312. Because the region consisted of ~100 amino acid residues, we prepared 94 octapeptides of which N termini were shifted in every amino acid from 212 to 305 of HSP90α. ELISA with the octapeptide library was performed with all mAbs recognizing

### Table II

| HSP90 | mAbs | Immunogenic region |
|-------|------|-------------------|
| 48–196 | K41218 | Region I |
| 185–355 | K3701 | Region I |
| 216–285 | K41020 | Region I |
| 216–312 | K41233 | Region I |
| 290–312 | K3729 | Region I |
| 313–458 | K41016 | Region II |
| 533–603 | K3725A | Region II |
| 535–719 | K3705 | Region II |
| 604–732 | K41007 | Region II |

* Isof orm specificities of mAbs are presented as described in Table I.
* a The regions recognized by multiple mAbs are tentatively designated Regions I and II.
* b Distinct from Region II, the boundary of which (702–716) was defined in Figs. 4 and 5.
* c The isof orm specificity remains to be determined.

Region I except K3701 and K3716, which did not react with the α isof orm (Table I). As shown in Fig. 2, two-thirds of the mAbs (15/23) revealed single binding peaks at their respective positions; and accordingly, their epitopes were clearly defined. On the other hand, two binding peaks were observed with K3725D, K3738, and K41331; three peaks with K41110 and K41116C; and four major peaks with K3729 (Fig. 2, c and d). No bindings were observed with K41102 and K41028 (Fig. 2d). With four mAbs (K3725D, K41110, K41116C, and K41331) among those that revealed multiple peaks, the peaks were close together (Fig. 2, c and d). This suggested that more than eight amino acids were involved in recognition of the mAbs and that several combinations of amino acids less than eight residues were sufficient for the bindings.

K3738, an HSP90β-preferential mAb, revealed two peaks relatively distant from each other (Fig. 2d, open squares). However, the two binding regions (DDEAEKEKEKEEE, 232–244 and EEEKKEKEKEEDK, 242–255) could not be separated when all peptides interacting with the mAb were aligned. That is, the last peptide (amino acids 237–244) forming the first peak overlapped the first peptide (242–249) forming the second peak. In addition, it should be remembered that the hybridoma producing K3738 was derived from a mouse immunized with the β isof orm. HSP90β has a three-amino acid deletion corresponding to 238–240 of HSP90α. Moreover, the two binding regions are considerably similar to each other; and thus, we postulate that either the deletion, the similarity between the two regions, or both explain the two binding peaks of K3738. Accordingly, we prudentely propose that the epitope of K3738 is localized within amino acids 232–255. Unexpectedly, another HSP90β-preferential mAb, K3729, bound to various peptides at completely distinct positions, i.e. DKEVSDDDEAEK (227–238), IEDVGSDEEEKK (258–270), EKYIDQEEELN (282–291), and PDDTNEEYG (301–310) (Fig. 2d). Again, there are sequence similarities between amino acids 227–238 and 258–270 and between 282–291 and 301–310. If not all, this may explain the multiple binding peaks of K3729.

The results of ELISA with the octapeptide library clearly showed that the boundaries of Region I were amino acids 227
and 310. In addition, three sites, designated Sites Ia, Ib, and Ic, emerged as the most immunogenic regions. Site Ia (247–257) was recognized by K41116A, K41122A, K41320, and K41110; Site Ib (263–270) was recognized by K41020, K41107, K41123, K41241, and K41315; and Site Ic (291–304) was recognized by K3720, K41110, K41116C, K41220, K41322, K41331, K41338, and K41122B. The epitopes of K41346 and K3738 overlapped with Site Ia. K3729 recognized four sites (227–238, 258–270, 282–291, and 301–310) in which overlapping of the second site with Site Ib, the third one with the epitope of K3725D (280–291), and the fourth one with Site Ic occurred. These results indicate that the mAbs recognizing Region I bound to one of Sites Ia–Ic or to the regions that overlapped with them (Fig. 3).

Analysis of the Recognition Sites at the C-terminal Region (Region II)—We also characterized the recognition sites at the C-terminal region by immunoblotting analyses of both chimeric proteins and deletion mutants. First, because the mAbs recognizing this region were specific or highly preferential to either one HSP90 isoform or the other (Table II), we investigated the recognition sites with chimeric proteins of the two isoforms (Fig. 4a). Although all chimeric proteins migrated at the same position on SDS-PAGE, they were clearly distinguished by the immunoblotting. K41009, an HSP90α-specific mAb, bound to HSP90α535–700/a701–732 but did not do so to the counter chimera (Fig. 4b, lanes 3 and 4). On the other hand, K3705, an HSP90β-specific mAb, bound to HSP90α535–700/b701–732 (Fig. 4c, lane 3). Thus, K3705 and K41009 recognized amino acids 701–732 of their respective isoforms. K3714, K3725B, and K41320 also recognized the same region of their respective isoforms (data not shown). Furthermore, when the bindings of K3705, K3714, and K3725B to amino acids 535–719 of HSP90β are taken into account (Table II), it seems reasonable to further narrow the recognition site of the HSP90β-specific mAbs to amino acids 701–719.

The C-terminal recognition sites of the HSP90α-specific mAbs were further investigated with the deletion mutants (Fig. 5a). Immunoblotting analysis with K41009 demonstrated its binding to HSP90α542–720, but not to HSP90α542–697 (Fig. 5b, lanes 4 and 5). An identical result was obtained with K41007 (data not shown). Taken together with the results of immunoblotting of the chimeric proteins (Fig. 4b), we concluded that K41009 and K41007 recognized amino acids 701–720. Therefore, two HSP90α-specific mAbs (K41007 and K41009), two HSP90β-specific mAbs (K3705 and K3725B), and a mAb highly preferential to HSP90β (K3714) should recognize identical or very close sites within amino acids 701–720. Isoform-specific bindings of the mAbs that bound to Region II further suggest that the highly substituted amino acids (702–716) correspond to their epitope (Fig. 4a). We tested this possibility by use of the phage display system. We obtained a phage that specifically accumulated after four pannings with an HSP90α-specific mAb, K41007. The heptapeptide sequence expressed on the phage surface was WVADTSY, of which ADTS is similar to the ADDTS (705–709) of HSP90α. Therefore, it is most likely that the highly substituted site at amino acids 702–716 contains the epitope recognized by K41007.

According to the epitope mapping described above, it was obvious that the recognition site of K3725A (533–603) was distinct from Region I. Hence, we obtained three mAbs (K3725A, K41016, and K41218) that recognized respective regions distinct from Regions I and II. We also determined the epitope for AC88, a mAb bound to HSP90 of various species (17), because its epitope is not precisely defined, but is reported to reside in the C-terminal region (18). We found that the epitope for AC88 was located within 604–689, where no mAbs developed herein bound (data not shown).

Limited Proteolysis of HSP90α—We previously investigated the protease sensitivity of the C-terminal 200 amino acids of HSP90α, and determined the chymotryptic sites of HSP90α at Tyr627–Met628 and Met628–Ala629 and the thrombin sites at Lys616–Ala616 and Arg620–Asp621 (5). We here investigated the domain structures of the full-length form of HSP90 by limited proteolysis. H6HSP90α, recombinant HSP90α tagged with a
dodecapeptide, was used in this study because the peptide was small enough not to affect the electrophoretic mobility of the protein, and the recombinant protein exists as a dimer as does the native protein (19).

H\textsubscript{6}HSP90\textsubscript{a} was purified to near homogeneity by affinity chromatography (Fig. 6, lane 1). The N-terminal sequencing showed that the major contaminant with a 20-kDa fragment was associated and copurified with intact H\textsubscript{6}HSP90\textsubscript{a}. Cleavage at the lowest trypsin concentration generated 15-, 50-, and 80-kDa fragments (lane 2). Subsequently, 27- and 40-kDa fragments appeared at intermediate enzyme concentrations (lanes 3 and 4). At higher trypsin concentrations, 22-, 32-, and 33-kDa peptides appeared (lanes 5–10). We found that the degradation profile of chymotrypsin treatment was similar to the tryptic cleavage profile (data not shown).

To address the origins of the proteolytic fragments, we determined their N-terminal amino acid sequences. Despite the appearance of many proteolytic peptides, only four N termini were detected: Glu\textsuperscript{401}, Ala\textsuperscript{616}, Asp\textsuperscript{621}, and the N terminus of the recombinant protein (Table III). The 15-kDa species was a mixture of two peptides starting at Ala\textsuperscript{616} and Asp\textsuperscript{621}, the same as that found in our previous study of thrombin cleavage (5). We also found that liberation of the N-terminal dipetide (MR, 12–11) caused alteration of the electrophoretic mobility from 32 to 33 kDa (Table III).

To determine the location of the proteolytic fragments, we first analyzed the C-terminal amino acid sequences of each of the fragments by automated Edman degradation. The results are summarized in Table III. The N-terminal 32/33-kDa part is the most protease-resistant because of the sensitivity of the immunoblotting analysis, several additional bands, e.g. 42- and 47-kDa ones (Fig. 7), were apparently detected, while they were faint by Coomassie staining (Fig. 6). These results indicate that Region I is truly localized in the C-terminal, 18-kDa portion of the N-terminal 50-kDa domain structures and immunogenic regions of HSP90.

![Fig. 4. Epitope mapping of Region II with chimeric proteins of HSP90alpha and beta.](image)

![Fig. 5. Immunoblotting analysis of Region II with the C-terminal deletion mutants of HSP90alpha. A series of C-terminal deletion mutants of HSP90alpha were separated by SDS-PAGE and stained with Coomassie Blue (a) or by immunoblotting with K41009 (b). Lanes 1, GST-HSP90alpha535–732; lanes 2, GST-HSP90alpha542–732; lanes 3, GST-HSP90alpha542–728; lanes 4, GST-HSP90alpha542–720; and lanes 5, GST-HSP90alpha542–697.)](image)
fragment. Taken together with these findings, the origins of tryptic peptides and the 20-kDa one, and their relationship to Region I, were deduced as shown in Fig. 8.

**DISCUSSION**

In this study, we developed 33 anti-HSP90 mAbs, including $\alpha$ and $\beta$ isoform-specific ones (Table I), and determined the epitopes recognized by them (Table II and Figs. 3 and 9). We confirmed that all mAbs specifically recognized HSP90 endogenously expressed in human salivary gland adenocarcinoma (22) and T47D cells, and we found that most of the mAbs cross-reacted with rat, mouse, and rabbit HSP90s. 2 This is not surprising in view of the evolutional conservation of HSP90. To our knowledge, this is the first report of the development of isoform-specific mAbs against HSP90. Since the epitopes for the mAbs have been characterized, these antibodies should be valuable to investigate the structure and function of HSP90 isoforms.

We found that two regions (Regions I and II) confer most immunogenicity of HSP90. Only three mAbs (K3725A, K41016, and K41218) developed in this study and AC88 recognized respective regions different from Regions I and II (Table II). Region I was defined to amino acids 227–310 by ELISA with the octapeptide library. Notably, there is a charged region (223–289) containing six amino acid substitutions of opposite net charges at 241, 242, 247, 257, 271, and 273 (Fig. 9). These characteristics strongly suggest that amino acid substitutions are permissive only when charged properties are maintained. Among anti-HSP90 mAbs reported previously, BF4 (6) also recognizes the charged region (18). This charged region is proposed to form an $\alpha$-helical structure that the polyglutamic acids of which are stabilized by ionic interactions with lysines (23). In HSP90, there is another charged region at amino acids 534–585 (23), suggesting its exposure to the outer surface. However, only K3725A recognized this region among all mAbs developed (Table II), indicating that this region does not possess a strong immunogenicity.

The existence of three immunogenic sites (Sites Ia–Ic) in Region I was demonstrated. The characteristics of Sites Ia and Ib are substantially different from those of Site Ic: the amino

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**TABLE III**

*N-terminal sequences of proteolytic fragments of H$_6$HSP90a*

Numbers represent amino acid residues of HSP90a. Twelve amino acids written in small letters (mrgshhhhhhg, −12−1) are derived from the vector and precede the first Met of HSP90a. Arrows indicate proteolytic sites.

| Peptide Detected amino acids Corresponding sequence |
|---|---|
| 15$^a$ AQALRDSTMGYMAAKK K | $\alpha_4$,AQALRDSTMGYMAAKK |
| 20$^a$ DNSTMGYMAAKKHLEIN R | $\alpha_2$,DNSTMGYMAAKKHLEIN |
| 21 IMKIDEKKEVVKVVSNR K | $\alpha_5$,IMKIDEKKEVVKVVSNR |
| 22 EMLQSKILKVR R | $\alpha_6$,EMLQSKILKVR |
| 27 EMILQKILKVR R | $\alpha_7$,EMILQKILKVR |
| 32 MRGSHHHHHGSMPEEM | m$_{12}$rgshhhhhhgsmpee |
| 33 GSHHHHHGSMPEETQT | m$_{12}$rgshhhhhhgsmpee |
| 40 MRGSH | m$_{12}$rg |
| 50 MRG | m$_{12}$rg |
| 80 MRGSH | m$_{12}$rg |

$^a$ A mixture of two peptides starting at Ala$^{616}$ (40.2%) and Asp$^{621}$ (59.8%).

$^b$ A peptide copurified with $H_6$HSP90a.
acid sequences of the former two are highly charged, and sub-
stituted between HSP90α and HSP90β; and hence, the mAbs recog-
nizing these two sites are specific or highly preferential to
HSP90α. We could not define the epitopes of K3701 and K3716,
mAbs recognizing Region I and specific or highly preferential to
HSP90α. However, it seems reasonable to postulate that their
recognition sites are also located at Site Ia or Ib. In contrast,
the amino acid sequence of Site Ic is less charged and is com-
pletely conserved between the isoforms. Hence, the mAbs
bound to Site Ic equivalently recognize the two isoforms (Fig.
3). Prediction of the secondary structures indicates that the
charged region (223–289) carrying Sites Ia and Ib, and the
region of amino acids 321–345 form α-helical structures and
that their intermediate region (290–320) carrying Site Ic (291–
304) may form a β-turn or coiled structure (23). The strong
immunogenicity of the α-helical structure composed of the
charged amino acids and the adjacent β-turn or coiled structure
strongly suggests the protruding of these conformations into
the aqueous environment.

We found a second immunogenic site (Region II) at the C-
terminal region. The C-terminal region (amino acids 699–732)
has hydrophilic characteristics although the degree is less than
that of Region I (Fig. 9). This difference is mainly due to the
absence of basic amino acids except Arg at 702–716 and the presence
of several hydrophobic amino acids. Nevertheless, it is important
to note that acidity of the five amino acid residues at 702, 706,
707, 714, and 723 is conserved in the replacements between the
isoforms. Considering the isoform-specific properties of the
mAbs recognizing Region II, the highly substituted amino acids
at 702–716 are the most probable recognition site of the mAbs.
In fact, we selected a heptapeptide with an affinity for an
HSP90α-specific mAb (K41007). The sequence (WVADTSY)
was similar to amino acids 705–709 of HSP90α (Fig. 9). Thus,
Region II is ascribed to amino acids 702–716 (Fig. 9). HtpG, an
E. coli homolog of mammalian HSP90, has 37% amino acid homology with human HSP90α (13). Interestingly, Region I,
except Site Ic, and Region II are highly substituted between
HSP90 isoforms and are even deleted in HtpG. These facts
strongly suggest that these regions are dispensable for the
function of HSP90.

We previously reported that bacterially expressed
HSP90α1–47/290–732, i.e. HSP90α with Sites Ia and Ib de-
leted tends to form a self-oligomer and is able to form a het-
eromic complex with the estrogen receptor (24). In contrast,
HSP90α possessing Sites Ia and Ib (HSP90α1–312) neither
forms an oligomer nor interacts with the receptor (24). These
results indicate that HSP90α, with the major part of Region I
deleted, acquired enhanced activity for associating with the
estrogen receptor as well as its self-oligomerizing activity. A
recent study indicates that the oligomerization of HSP90 is
closely related to its chaperone activity (15). Thus, we suspect
that one possible role of Region I is to suppress the oligomer-
ization activity of HSP90 under nonstressed conditions.

We further analyzed the domain structures of HSP90 by
means of limited proteolysis. The N-terminal sequencing analy-
sis demonstrated that cleavages occurred only at a few sus-
cetable sites at Arg424–Glu427, Lys428–Ala430, and Arg620–Asp621.
We also showed that mAbs recognizing Region II recognize
at lower protease concentrations. The former site has not been
recognized before because our previous study focused on the
C-terminal dimer-forming region (5). We found that, even at
higher enzyme concentrations, trypsin attached several partic-
ular sites. Immunoblotting analysis confirmed that Region I
was localized in the protease-sensitive region.

Based on the present results, we finally propose a model for
the domain structures of HSP90 (Fig. 8). HSP90 is composed
of three domains, Domains A–C, corresponding to amino acids
1–400, 401–615, and 621–732, respectively. Further, the di-
vision of each domain into two subdomains is suspected from the
differences in proteolytic susceptibility and immunogenicity.
The subdomain structures of Domains B and C are also sug-
gested from our previous study, demonstrating that chymo-
trypsin cleaves at Leu261–Val264, Tyr267–Met268, and Me265–
Ala266 bonds (5). The deletion in HtpG corresponding to amino
acids 696–732 further suggests the structural and functional
differences between amino acids 604–700 and 701–732. The
characteristics of each region are as follows: subdomain A-I is
protease-resistant; subdomain A-II is protease-sensitive, is
abundant in charged amino acids, and carries the most immu-
nogenic region of HSP90 (Region I), which may be exposed to
the outer surface of the molecule; subdomain B-I is resistant to
proteolysis; subdomain B-II interacts with subdomain C-I of
another HSP90 subunit, and this interaction mediates the
dimerization of HSP90; and subdomain C-II carries the second
immunogenic region (Region II) and is not involved in dimer
formation. Although the validity of this model, especially as to
the subdomain structures, should be tested by functional and
chemical studies, we believe that this provides an appropriate
working model for investigating the structure and function of
HSP90.

Acknowledgments—We are grateful to Dr. Y. Ohara-Nemoto for
discussion and critical reading of the manuscript.

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FIG. 9. Comparison of the amino acid sequences at Regions I
1 and II between HSP90α and HSP90β. The amino acid sequences
around the immunogenic domains are compared between HSP90 iso-
forms. Regions I and II are indicated by arrows. Hatched bars represent
the regions deleted in HtpG. Open bars represent the charged regions.
a, sites Ia–c are boxed. Six amino acid replacements between Lys and
acidic amino acids are indicated by arrowheads. b, asterisks indicate the
sequence (ADTSTY, 705–709) similar to that of a phage peptide
boxed, sites Ia–c are
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