Oligomeric forms of the 90-kDa heat shock protein

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Two isoforms of the 90-kDa heat shock protein, HSP90 α and HSP90 β , are present in the cytosol of mammalian cells. Analysis by polyacrylamide gel electrophoresis under nondenaturing conditions (native PAGE) revealed that HSP90 α predominantly exists as a homodimer and that HSP90 β is present mainly as a monomer [Minami, Kawasaki, Miyata, Suzuki and Yahara (1991) *J. Biol. Chem.* **266**, 10099–10103]. However, only the dimeric form has been observed under other analytical conditions such as gradient centrifugation. In this study, therefore, we investigated native forms of HSP90 by use of immunochemical techniques with isoform-specific monoclonal antibodies recently developed in our laboratory. Glycerol gradient centrifugation at the physiological salt concentration as well as native PAGE analysis of rat liver cytosol revealed oligomeric forms of HSP90 α sedimenting at 8–10S as predominant ones. On the other hand, the glycerol gradient centrifugation revealed multiple forms of HSP90 β oligomers sedimenting at 6–12S. All of the HSP90 β

oligomers, however, migrated at 100-kDa monomer and 190-kDa dimer positions on native PAGE. A novel two-dimensional double native PAGE revealed that the entity was converted from the HSP90 β dimer to monomers during the electrophoresis. The same PAGE further revealed that the HSP90 α oligomer also dissociated into dimers during the electrophoresis. Full-length form of bacterially-expressed human HSP90 α migrated as dimers, but a considerable amount did not penetrate into the gel under native PAGE conditions, indicating the existence of oligomeric forms. Electrophoretic studies of deletion mutants of HSP90 demonstrated that the C-terminal 200 amino acids were capable of forming oligomers. Taken together, we conclude that both of the HSP90 isoforms predominantly exist as oligomeric forms in the cytosol even under unstressed conditions but that they artificially dissociate into smaller forms when subjected to native PAGE.

INTRODUCTION

The 90-kDa heat shock protein (HSP90) is a molecular chaperone functioning in the cytoplasm of eukaryotic [1,2] and prokaryotic cells [3]. In mammalian cells, there are at least two HSP90 isoforms, HSP90 α and HSP90 β , encoded by separate genes. The amino acid sequence of HSP90 α is 85 % (human [1,2]); 90 % (yeast [4]) homologous to that of HSP90 β , and either one of the isoforms is indispensable for the growth of yeast cells at high temperature [4]. It has been reported that HSP90 α predominantly exists as a homodimer and that HSP90 β occurs mainly as a monomer [5]. The dimeric structure of HSP90 is mediated by the C-terminal 191 amino acids; and, accordingly, amino acid substitutions at this C-terminal region between α and β isoforms are responsible for the impeded dimerization of HSP90 β [6].

The native forms of HSP90 isoforms described above, i.e. dimeric HSP90 α and monomeric HSP90 β , are, however, not compatible with the findings of several studies. For instance, dimeric integrity of HSP90 appears to be essential for survival of yeast cells [7], despite the monomeric structure of HSP90 β [5]. In addition, HSP90 β as well as HSP90 α is a constituent of the nontransformed oligomeric form of the glucocorticoid receptor [8], but the dimeric form alone is associated with the receptor [9]. The monomeric form of HSP90 was solely detected by PAGE under nondenaturing conditions (native PAGE) [5], but was not observed by other analytical techniques such as gradient centrifugation and size exclusion HPLC [9–12]. Furthermore, by affinity chromatography with an anti-receptor monoclonal anti-

body (mAb), Bresnick et al. [13] detected 4–7 mol of HSP90 molecules per mol of the nontransformed glucocorticoid receptor.

Monoclonal antibodies against HSP90 have been produced by several researchers [14,15]. However, none of these mAbs distinguish between HSP90 α and HSP90 β . For the analysis of the expressional regulation and functional difference between the isoforms, such antibodies discriminating between the two HSP90 isoforms are sorely needed. We recently developed a library of mAbs from mice immunized with bacterially expressed human HSP90 α and HSP90 β as antigens [16]. Among all 33 anti-HSP90 mAbs, ten mAbs specifically reacted with HSP90 α and three with HSP90 β . In this study, we investigated the native forms of HSP90 isoforms in the rat liver cytosol by use of these mAbs. The results of this study clearly show the reason why the native forms of HSP90 varied among the studies reported. That is, HSP90 predominantly exists as oligomeric forms; and the oligomers artificially dissociate into smaller forms under electrophoretic conditions. The oligomerization as well as the dimerization is mediated by the C-terminal 200 amino acids of HSP90.

MATERIALS AND METHODS

Materials

A plasmid, pQE10, was purchased from Qiagen (Chatworth, CA, U.S.A.). Talon metal-affinity resin was purchased from Clontech Lab. Inc. (Palo Alto, CA, U.S.A.). Alkaline phosphatase-conjugated goat anti-mouse IgG and IgM were purchased from Bio-Rad (Richmond, CA, U.S.A.) and EY Lab. Inc.

Abbreviations used: HSP90 α and β , the α and β isoforms of the 90-kDa heat shock protein, respectively; H₆HSP90, HSP90 tagged with a histidine hexamer; mAbs, monoclonal antibodies; native PAGE, PAGE under nondenaturing conditions; 2DDN/PAGE, two-dimensional double native PAGE; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, Nitro Blue tetrazolium.

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(San Mateo, CA, U.S.A.), respectively. Bovine serum albumin (minimum 98 % pure and essentially immunoglobulin free) was purchased from Sigma. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue tetrazolium (NBT) were obtained from Promega (Madison, WI, U.S.A.) or Kirkegaard & Perry (Gaithersburg, MD, U.S.A.). All other reagents were of analytical grade.

Anti-HSP90 mAbs

K41116A and K3705 were used as HSP90 α - and HSP90 β -specific mAbs, respectively [16]. The former is an IgG_{2b}; and the latter, an IgM. The isoform-specificities of the mAbs were extensively examined by ELISA and immunoblotting analysis with bacterially-expressed human HSP90 isoforms. Both mAbs reacted with human [16], rat, and rabbit (data not shown) HSP90. The epitope of K41116A was defined to be amino acids 247–257 of HSP90 α ; and that of K3705, amino acids 694–708 of HSP90 β [17].

Preparation of the cytosol

The cytosol was obtained by homogenization of male Wistar rat liver in 5 vol. of 20 mM Tris/HCl [pH 7.3], 1 mM EDTA, 1 mM dithiothreitol, and 50 mM NaCl with protease inhibitors (20 μ g/ml each of leupeptin, pepstatin, and soybean trypsin inhibitor) and subsequent centrifugation at 150 000 *g* for 1 h at 4 °C. The clear supernatant was used as the cytosol.

Expression and purification of the full-length and deleted forms of HSP90

The expression plasmids pH₆HSP90 α , carrying the full-length form of human HSP90 α (amino acids 1–732), and pH₆HSP90 α 533–732, carrying the C-terminal 200 residues, both tagged with a histidine hexamer at their N-terminus, were described previously [18]. The deleted forms (pH₆HSP90 α 1–47/290–732, pH₆HSP90 α 1–312 and pH₆HSP90 α 1–47/459–732) were prepared by restriction cleavages of pH₆HSP90 α at appropriate restriction sites followed by in-frame ligation. For the construction of pH₆HSP90 β 521–724, the *ScaI*–*SalI* fragment of human HSP90 β cDNA (a gift of Dr. S. Hickman, Washington University) carrying amino acids 521–724 was inserted in-frame into a filled-in *BamHI*/*SalI* site of pQE10. The recombinant proteins designated H₆HSP90 α and that with corresponding amino acids were expressed as described [18] and purified by Talon metal-affinity chromatography according to the manufacturer's protocol in the presence of 10 mM imidazole.

Glycerol gradient centrifugation

Linear glycerol gradients (5 ml) of 5–20 % were prepared in 20 mM Tris/HCl (pH 7.3), 1 mM EDTA, and 0.5 mM dithiothreitol containing 0.13 M NaCl. An aliquot (0.25 ml) of the cytosol was layered onto the gradient and centrifuged at 200 000 *g* for 13 h at 4 °C. Five-drop fractions were collected from the top of the gradient. Ovalbumin (3.5 S), bovine serum albumin (4.6 S), horse IgG (6.6 S), and bovine catalase (11.3 S) were centrifuged in parallel as sedimentation markers.

PAGE and immunoblotting

Proteins were subjected to electrophoresis under denaturing conditions (SDS/PAGE) of Laemmli [19] or nondenaturing conditions (native PAGE) according to the method of Davis [20]. Native PAGE was performed at 4 °C, but later we found that

performance at room temperature gave the same results. Separated proteins were stained with Coomassie Brilliant Blue or transferred to a PVDF membrane (Millipore). After blocking of the residual binding sites with 5 % skim milk (Difco Lab.) in 50 mM Tris/HCl (pH 7.6) containing 0.15 M NaCl (TBS), the membrane was incubated with anti-HSP90 mAbs (2 μ g/ml) at 25 °C for 2 h in TBS containing 0.1 % Tween-20. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM was used as the second antibody at a 1:5000 dilution in the same buffer. After having been washed with TBS containing 0.1 % Tween-20, blots were visualized by incubation with BCIP and NBT (Promega). As molecular markers under nondenaturing conditions, ovalbumin (45 kDa), bovine serum albumin (monomer, 66 kDa; dimer, 132 kDa; trimer, 198 kDa), and horse catalase (240 kDa) were run in parallel.

ELISA

Quantitative analysis of HSP90 β was performed by ELISA. Aliquots of the fractionated samples of gradient centrifugation were adsorbed to a 96-well titre plate (Falcon) for 2 h at 25 °C. After blockage of residual binding sites with TBS containing 0.5 % skim milk (Difco Lab., Detroit, MI, U.S.A.), 50 μ l of K3705 (0.5 μ g/ml) was incubated at 25 °C for 2 h. Following incubation with alkaline phosphatase-conjugated anti-IgM (1:5000 dilution, 50 μ l) as the second antibody as above, the colour was developed with BCIP and NBT in a soluble buffer system (Kirkegaard & Perry Lab.). The absorbance at 600 nm was measured following 1 h incubation at 30 °C.

Two-dimensional double native PAGE (2DDN/PAGE)

Samples were initially electrophoresed under nondenaturing conditions at a 7.5 % polyacrylamide concentration. The sample lane was then stripped and layered on the second-dimension native gel of identical compositions. After the second-dimension electrophoresis, proteins were either stained with Coomassie Brilliant Blue or subjected to immunoblotting as above.

RESULTS

Native PAGE of HSP90 isoforms in rat liver cytosol

The rat liver cytosol was subjected to native PAGE, and the HSP90 α isoform was detected by immunoblotting with the HSP90 α -specific mAb, K41116A. As a result, a major band was found at the position above 190 kDa (Figure 1a, lane C, indicated by the asterisk). Its migrating position and broad distribution strongly suggested the existence of multiple forms of oligomers. Additionally, a faster-migrating species was observed as a 110-kDa band, presumably representing a monomeric form. On the other hand, when the immunoblotting was performed with the HSP90 β -specific mAb, K3705, a 100-kDa band was predominant, and a faint band was observed at 190 kDa (Figure 1b, lane C). The 100- and 190-kDa bands probably correspond to monomeric and dimeric forms of HSP90 β , respectively. These results were basically consistent to the previous findings [5] that HSP90 β has an impeded dimerizing ability compared with HSP90 α . However, an apparent difference was noted: we found that HSP90 α predominantly existed as oligomers rather than as a dimeric form.

Analysis of the native forms of HSP90 isoforms by glycerol gradient centrifugation

The cytosol was first subjected to the glycerol gradient centrifugation under the physiological salt concentration, and frac-

Figure 1 Glycerol gradient centrifugation of HSP90 isoforms

Rat liver cytosol (0.25 ml) was layered onto a 5–20% glycerol gradient and centrifuged at 200 000 g for 13 h at 4 °C. Following fractionation from the top, an aliquot (20 μ l) was subjected to native PAGE and immunoblotting as described in Materials and methods. HSP90 α (a) and HSP90 β (b) were visualized with K41116A and K3705, respectively. Lanes 6–40 represent numbers of the fractions of the gradient. In (a) the asterisk shows the position of oligomeric species. (c) An aliquot (25 μ l) of the fractionated sample of the gradient was adsorbed to a well of a 96-well titre plate, and the quantity of HSP90 β was determined by ELISA with the HSP90 β -specific mAb, K3705, at absorbance of 600 nm. Sedimentation coefficients of the marker proteins are indicated at the top.

tionated samples were analysed by native PAGE followed by the immunoblotting with the isoform-specific mAbs. As shown in Figure 1a, HSP90 α sedimented at a wide range of 4–10 S, mainly at 4–6 S (fractions 10–18) and 8–10 S (fractions 22–28). Analysis by native PAGE revealed that the 4–6 S species consisted of the 110- and 190-kDa species. The latter species had been hidden behind the intense band of the oligomeric forms in the cytosol (Figure 1a, lane C). The previous results that the HSP90 dimer sediments at 5.8–6.2 [9–12] further supported the dimeric configuration of the 190-kDa species. On the other hand, the 8–10S species has not been reported to date. Its large sedimentation coefficient on centrifugation and the retarded migration profile compared with that of the 190-kDa dimer on native PAGE indicated a tetrameric or further oligomeric structures. Moreover,

bands larger than 240 kDa were observed at fractions 32–40 with the naked eye, but were not of sufficient density to be visualized in the figure. The quantity of these species is presumably underestimated because of incomplete electroblotting of large-molecular-mass proteins.

Immunoblotting analysis with the HSP90 β -specific mAb revealed that HSP90 β sedimented at a broad range of 6–12 S (fractions 16–34 in Figure 1b), implicating its heterogeneous characteristics. ELISA of the same gradient fractions confirmed this notion: a major peak at 7.5 S (fractions 20 and 21) and several additional peaks with larger sedimentation coefficients (8.5, 10, 10.8 and 11.5 S at fractions 24, 28, 30, and 32, respectively; Figure 1c). Notably, there was no HSP90 β species with the sedimentation coefficient smaller than 6.6 S, implying the entire absence of monomeric and dimeric forms. However, immunoblotting analysis revealed the 100-kDa monomeric and 190-kDa dimeric forms irrespective of their sedimenting positions (Figure 1b). These results strongly suggested that HSP90 β as well as HSP90 α existed as oligomers under certain conditions and that the molecular configuration of HSP90 β was altered by the analytical procedures. Thus, there are two alternative possibilities for the native structure of HSP90 β : One is that HSP90 β predominantly exists as oligomers and dissociates into smaller forms under native PAGE conditions; or inversely, HSP90 β predominantly exists as a monomer in the cytosol and oligomerizes during gradient centrifugation.

Two-dimensional double native PAGE

During the course of native PAGE analysis, we noticed the presence of some blots tailing between the monomeric and dimeric bands of HSP90 β (Figure 1b, fractions 16–34). We suspected that the tailing represented an entity converting between monomeric and dimeric forms during the electrophoresis. If so, the species should be identifiable as a band(s) distinct from monomeric and dimeric forms by the second dimension of native PAGE following the ordinary first dimension of native PAGE. We designated this method as 2DDN/PAGE.

Although various separation patterns of the oligomeric proteins on 2DDN/PAGE are to be expected, a typical example in which only monomeric and homodimeric forms of a protein are present will be considered. The first case is that the two forms are stable, not interconverting under electrophoretic conditions. In this case, the monomer and the dimer should be aligned in a straight line extrapolated from the loading point (Figure 2a). On the other hand, when the two forms are exchangeable during electrophoresis, the following three cases are expected: monomeric and dimeric forms are readily exchangeable with each other and are in a certain equilibrium (Figure 2b); monomers dimerize during electrophoresis and the dimer does not dissociate (Figure 2c); vice versa, the dimer dissociates into monomers and the reverse process does not occur (Figure 2d). In these three cases, the monomer and dimer spots may be connected with characteristic lines that represent the monomer or the dimer newly occurring during the electrophoresis (Figures 2b–2d). Additionally, it is possible to assume a pattern that the loading origin and two spots are unable to be connected by a straight line (Figure 2e). This could indicate that HSP90 is associated with heterologous proteins or that the mAbs nonspecifically bind non-HSP90 molecules. As schematically illustrated (Figure 2), all of these possibilities would be expected to be distinguishable from each other.

As a control experiment, bovine serum albumin was subjected to 2DDN/PAGE. The commercial preparation of this protein is

Figure 2 Theoretical migration patterns of the monomeric and dimeric proteins on 2DDN/PAGE

The potential migration patterns of monomeric and dimeric forms of a protein on 2DDN/PAGE, i.e. native PAGE on first and second dimensions, are schematically illustrated. (a) The two forms of a protein do not interconvert during electrophoresis. (b) The two forms rapidly interconvert to each other during electrophoresis. (c) The monomeric form dimerizes during electrophoresis. (d) The dimeric form dissociates into monomers. (e) The two species are unrelated. X indicates the position of loading. M and D are monomeric and dimeric forms of a protein, respectively.

a mixture of a monomer, dimer, trimer, and further oligomers [6]. As shown in Figure 3a, all spots were aligned in a straight line extrapolated to the loading point as assumed in Figure 2a. Accordingly, it was clearly shown that all forms of albumin did not interconvert to other forms under electrophoretic conditions.

We then subjected the rat liver cytosol to 2DDN/PAGE and then specifically immunoblotted the HSP90 isoforms. The dimeric form of HSP90 α was predominant, and monomeric and oligomeric forms were present as minor species on 2DDN/PAGE (Figure 3b). However, the oligomeric form of HSP90 α was more abundant than the dimeric one at the first dimension of 2DDN/PAGE (Figure 3b, indicated at the left and the top by asterisks). This finding indicated that HSP90 α was predominantly present as oligomeric forms and dissociated into dimers during 2DDN/PAGE.

The 2DDN/PAGE pattern of HSP90 β was distinct from that of HSP90 α . Immunoblotting specific for HSP90 β revealed an L-shaped pattern starting at the dimer position and ending at the monomer one (Figure 3c). This pattern was exactly that predicted in Figure 2d. The horizontal and vertical blotting lines should represent the entities converting from the dimeric to monomeric forms during the first and second dimension native PAGE, respectively. Accordingly, 2DDN/PAGE clearly demonstrated that the tailing blot observed between the monomeric and dimeric bands (Figure 1b) represented the entity converting from the dimer to the monomer during the electrophoresis. Additionally, a species larger than the 190-kDa dimer was found as broad blots on the PAGE (Figure 3c, indicated by asterisks), implicating the presence of oligomeric forms of HSP90 β in the cytosol, which is inconsistent with the results of the glycerol gradient centrifugation. Therefore, we concluded that HSP90 β predominantly existed as a dimer or oligomers, which dissociated into monomers during the electrophoresis.

Localization of the oligomerization activity of HSP90 α

We here investigated the region sufficient for the oligomerization of HSP90. Firstly, we compared the dimer- and oligomer-forming abilities of the full-length and deleted forms of HSP90 α s by SDS

Figure 3 Two-dimensional double native PAGE of albumin and HSP90 isoforms

Fifty μ g of bovine serum albumin (a) or 400 μ g of the liver cytosol (b and c) was separated on 2DDN/PAGE as described in Materials and methods. The separated proteins were stained with Coomassie Brilliant Blue (a), or immunoblotted with HSP90 α -specific (K41116A) (b) or HSP90 α -specific mAb (K3705) (c). In (b) and (c), positions of the oligomeric forms are indicated by asterisks.

Figure 4 Oligomerization activities of various forms of HSP90

(a) The full-length and deletion forms of HSP90 are expressed in *E. coli*. Amino acids 521–724 of HSP90 β (number 6) are equivalent to amino acids 529–732 of HSP90 α . (b–d). The lane numbers correspond to the numbers in (a). The purified proteins were subjected to SDS (b) and native PAGE (c and d) and stained with Coomassie Brilliant Blue. Two, four, and twenty μ g of purified proteins were loaded onto each lane shown in (b), (c), and (d), respectively. Arrowheads indicate the positions of tetrameric and further oligomeric forms. Marker proteins of native PAGE (lane M in panels c and d) are: ovalbumin (45 kDa), monomeric (66 kDa), dimeric (132 kDa), and trimeric (198 kDa) forms of bovine serum albumin and catalase (240 kDa).

and native PAGE (Figure 4). As expected, H₆HSP90 α existed as a dimer (190 kDa) on native PAGE (Figure 4c, lane 1). We previously reported that the C-terminal 191 amino acids (542–732) were sufficient for the dimerization [6]. Accordingly, H₆HSP90 α 533–732 carrying the C-terminal 200 amino acids was expected to migrate at a dimer position (54 kDa) from the apparent molecular mass of the monomer (27 kDa). However, it actually migrated at a 70-kDa position (Figure 4c, lane 5). Nevertheless, we previously showed that the 70-kDa species is a dimeric form and that the retardation is simply due to the effect of the basic charges of the tagged peptide, which particularly affects the mobilities of small molecular-mass proteins [18]. On the other hand, H₆HSP90 α 1–312 (lane 3), in which the C-terminal 420 amino acids were deleted, entirely existed as a 45-kDa monomer.

Interestingly, in addition to the dimeric form, the species that hardly penetrated into the separation gel was observed for H₆HSP90 α and H₆HSP90 α 1–47/290–732 (indicated by arrowheads in Figure 4c, lanes 1 and 2). Moreover, a significant amount of H₆HSP90 α 1–47/469–732 (lane 4) and a lesser amount of H₆HSP90 α 533–732 (lane 5) existed as a tetramer and further oligomers (arrowheads in Figure 4c, lanes 4 and 5). When a larger amount of H₆HSP90 α 533–732 was loaded on the gel, tetrameric and hexameric forms were more evident (Figure 4d, lane 5). Its oligomeric characteristic was confirmed from the

sedimentation of the 70-kDa dimeric, 132-kDa tetrameric, and 198-kDa hexameric forms at 3, 5 and 6.5S positions, respectively, on glycerol gradient centrifugation (data not shown).

We also tested the oligomerization activity of H₆HSP90 β 521–724 which was equivalent to amino acids 529–732 of HSP90 α (Figure 4d, lane 6). It revealed a ladder pattern comprising monomer, dimer, trimer, and further oligomers. The appearance of the forms composed of odd molecules, which were not found for H₆HSP90 α 533–732, may reflect the impeded dimerization ability of HSP90 β under electrophoretic conditions. Thus, the C-terminal region of HSP90 β as well as that of HSP90 α possessed the oligomerizing activity.

DISCUSSION

In this study, we investigated the native forms of HSP90 isoforms. The mAbs specific for respective HSP90 isoforms developed recently [16,17] were used for identification of respective HSP90 isoforms. Thus, sample preparation used in this study was least manipulated among the studies that determined the native forms of HSP90. It is well known that HSP90 is associated with a variety of proteins in the cytosol. Heteromeric complexes between HSP90 and associating proteins would be randomly distributed on a gel of 2DDN/PAGE (Figure 2e). However, such bands were not observed. This implies that any one of the heteromeric complexes accounts for a minor population below the detection of the alkaline phosphatase detection system employed here, and that the self-oligomerized forms are predominant species of HSP90 isoforms.

We found that the oligomeric form of HSP90 α was predominant at the single dimensional native PAGE and was reduced at 2DDN/PAGE with a concomitant increase in the dimeric form (Figure 3b). Thus, the oligomeric forms of HSP90 α gradually dissociate into the dimer during the electrophoresis. Accordingly, the discrepancy as to the predominant form of HSP90 α between this and a previous study [5] appears to be due to the difference in the degree of dissociation of the oligomeric forms during native PAGE. On the other hand, glycerol gradient centrifugation revealed that there was essentially no HSP90 β corresponding to monomeric and dimeric forms and that HSP90 β was present as multiple forms of oligomers. 2DDN/PAGE clearly demonstrated that the dimeric form of HSP90 β converted to the monomeric form under native PAGE conditions. We could not directly demonstrate the conversion of HSP90 β from the oligomer to the monomers and dimers, whereas this conversion is reasonably postulated from the comparison of the structures under glycerol gradient and native PAGE conditions, i.e. oligomeric forms on the glycerol gradient and the monomeric form on native PAGE. We suspect that the conversion from the oligomer to the dimer of HSP90 reflects multiple steps of dissociation, which may be the reason why the converting forms were not clearly distinguished on 2DDN/PAGE, in contrast to the transition from the dimer to the monomer of HSP90 β . Taken together with these findings, we conclude that both HSP90 α and HSP90 β are present as oligomeric forms in the cytosol and convert to smaller ones during the electrophoresis.

A line of evidence has suggested the presence of the oligomeric structures of HSP90. For instance, immunological studies showed that 4–7 mol of HSP90 molecules is involved in 1 mol of the nontransformed glucocorticoid receptor [13]. Similarly, hormonal ligand-affinity chromatography resulted in copurification of 10-fold molar excess of HSP90 to one receptor molecule [21,22]. Nevertheless, one might raise the possibility that HSP90 isoforms artificially aggregate during the preparation step of the cytosol in a hypotonic buffer. An alternative method preparing

the cellular extract is the use of an isotonic buffer containing a detergent. In the cytosol prepared in the presence of Triton X-100, Lanks [23] reported the oligomerization of HSP90 to occur in a temperature-dependent manner at a temperature as low as 33 °C. Recent studies further indicate that the oligomerization of HSP90 at high temperature is involved in the process exerting the chaperone activity of HSP90 [24]. These findings strongly suggest that oligomerization is not an artifact, but an intrinsic property of HSP90. We therefore suppose that a basal level of oligomerization is exerted on HSP90 isoforms even under unstressed conditions.

At present, it is not known whether or not HSP90 is present as a homo-oligomeric form composed of respective isoforms or hetero-oligomeric form of the two isoforms. Glycerol gradient analysis revealed altered sedimentation profiles between HSP90 α and HSP90 β , indicating the homo-oligomeric structures. However, it is not completely ruled out that a small population of HSP90 isoforms forms a hetero-oligomer. In fact, Minami et al. [5] previously showed the existence of a heterodimer composed of HSP90 α and HSP90 β as a minor component. Further studies are needed to address this issue.

This study further demonstrated that the C-terminal 200 amino acids were sufficient for the oligomerization of HSP90 isoforms. The degree of oligomerization varied among the recombinant proteins expressed. Especially, heavy oligomerization occurred with H₆HSP90 α 1-47/290-732 (Figure 4c, lane 2). This indicates that some region(s) outside the C-terminal 200 amino acids may play a role in modulating the oligomerization of the molecule. For instance, Minami et al. [25] reported that the peptide corresponding to amino acids Lys-500-Ile-520 of mouse HSP90 α and the equivalent region of HSP90 β augment self-oligomerization of HSP90 at an elevated temperature.

Notably, native PAGE of H₆HSP90 β 521-724 revealed monomeric, trimeric, and pentameric forms in addition to dimeric and tetrameric forms (Figure 4d). This phenomenon faithfully reflects the difference in dimerizing activities of cytosolic HSP90 isoforms under electrophoretic conditions. This finding strongly suggests that the region responsible for oligomerization of HSP90 is distinct from that for dimerization, though both are colocalized within the C-terminal 200 amino acids. A study to identify the regions responsible for dimerization and oligomerization is now in progress.

Recently, three-dimensional structure of the N-terminal domain of yeast (residues 1-220) [26,27] and human HSP90 (residues 9-236) [28] has been reported. We have determined that this domain is most resistant to proteolysis [17]. Stebbins et al. [28] demonstrated that the domain contains the binding site for an antitumour agent geldanamycin, and supposed that the geldanamycin-binding site is in binding segments of an incompletely folded polypeptide chain. However, Prodromou et al. [27] identified the ATP/ADP-binding site at the same region and proposed that geldanamycin acts an ADP/ATP mimetic at the site. In their studies [26,27], the N-terminal domain was crystallized as a dimeric structure, of which opposing faces of the β -sheets define a potential polypeptide-binding cleft. They thus proposed that the N-terminal domain of HSP90 functions as a molecular clamp trapping a polypeptide in the cleft. However, a line of evidence of biochemical studies indicates that the dimer-forming activity of HSP90 family proteins primarily resides within the C-terminal region [5-7,18,29]. Moreover, the present study clearly demonstrated that the N-terminal domain essentially existed as a monomeric form (see Figure 4). In addition, the crystal structure of the domain reported by Stebbins et al. [28] is monomeric.

Further studies may be needed to address this issue. However,

one possible explanation is that the primary dimerization activity at the C-terminal domain facilitates the conformational change of the N-terminal domain into the dimeric structure possessing the polypeptide-binding cleft. There is such an example, that on interrelation between the DNA- and steroid-binding domains of steroid receptors: The dimerization of the DNA-binding domains of glucocorticoid [30] and oestrogen [31] receptors is greatly facilitated through the homodimeric structure of the C-terminal, steroid-binding domain. Moreover, we found that this function of the steroid-binding domain is partially substituted by fusion of the DNA-binding domain to a foreign protein capable of forming a dimer, e.g. glutathione *S*-transferase [32]. Thus, it is interesting to investigate whether the C-terminal domain of HSP90 could be replaced by a foreign dimeric protein.

In conclusion, this study demonstrated that HSP90 isoforms existed as multiple forms of oligomers in the cytosolic compartment, and that the other forms, such as dimeric HSP90 α and monomeric HSP90 β , were artificially produced as a result of the dissociation under electrophoretic conditions. Use of the specific mAbs enabled us to readily identify the respective HSP90 isoforms for the first time. The novel electrophoretic technique, 2DDN/PAGE, developed herein, should be useful for identifying the transition state between the monomer and the dimer of a protein.

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