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Dimerization Characteristics of the 94-kDa Glucose-Regulated Protein[1]

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The 94-kDa glucose-regulated protein (GRP94) is a member of the 90-kDa heat-shock protein (HSP90) family. In this study, we expressed the barley (*Hordeum vulgare* L.) GRP94 and the α isoform of human HSP90 (HSP90α) in *Escherichia coli* and compared their dimer-forming abilities. Native polyacrylamide gel electrophoresis revealed that GRP94 (amino acids 69-809) and the full-length form of HSP90α existed in the dimeric state. The C-terminal 326 amino acids of GRP94 or the C-terminal 200 amino acids of HSP90α were sufficient for the dimerization. Limited proteolysis of the C-terminal half of GRP94 with thrombin revealed a 16-kDa fragment, which was derived from the C-terminus of GRP94 through the cleavage of either the Arg710-His711 or the Arg735-Leu736 bond. These cleavage sites were nearly, if not completely, equivalent to the proteolyzed region of HSP90α. Their structural similarity prompted us to investigate, by use of a coexpression system, the possibility that the two proteins form a heterodimeric complex. A two-step affinity chromatography that specifically trapped only the complex revealed that the C-terminal 200 amino acids of HSP90α and the C-terminal 326 amino acids of GRP94 associated with HSP90α and GRP94, respectively. However, the C-terminal 326 amino acids of GRP94 failed to form a complex with HSP90α. In conclusion, these results indicate the similarity of the general dimeric conformation of the two HSP90 family member proteins, but show that the similarity is not sufficient to allow heterodimer formation.

Key words: amino acid sequence, coexpression system, dimerization, 94-kDa glucose-regulated protein, 90-kDa heat-shock protein.

The 90-kDa heat-shock protein (HSP90) is a highly conserved protein that is abundant in the cytoplasm of most prokaryotic and eukaryotic cells even under nonstressed conditions. In eukaryotic cells, there are two HSP90 genes; and two HSP90 isoforms, α and β, are expressed (*1, 2*). Either one of the HSP90 isoforms is required in high concentration for growth of yeast cells at high temperatures (*3*). The enhancement of HSP90α expression under stress conditions is more profound than that of HSP90β (*4*). To date, only one biochemical difference between the α and β isoforms has been reported (*5*): although HSP90α as well as HSP90β forms a homodimer, the dimer-forming ability of the α isoform is greater than that of β one. The disulfide bridges are not involved in the dimer formation (*6*). The loss of the 15-kDa C-terminal region of HSP90 causes the dissociation of the dimer (*7*). We recently reported that the two subunits of the HSP90α dimer are orientated in opposite directions and that the dimerization is mediated through the duplicate interactions of the C-terminal region (Ala629-Asp732) of one subunit and the adjacent N-terminal region (Val542-Lys615) of the other subunit (*8*).

In eukaryotic cells, a third HSP90-family protein, the 94-kDa glucose-regulated protein (GRP94), is localized in the lumen of the endoplasmic reticulum and is induced in response to the inhibition of protein glycosylation, such as occurs in glucose starvation and upon tunicamycin treatment (*9*). Like other endoplasmic reticulum-resident proteins, the N-terminal signal sequence and the C-terminal tetrapeptide, Lys-Asp-Glu-Leu, are essential for the retention of GRP94 in the endoplasmic reticulum (*10*). However, little is known about other biochemical properties of GRP94. For instance, it is not known whether or not this protein also exists as a dimer in the lumen of the endoplasmic reticulum.

We previously reported the cDNA sequence of barley GRP94, which shows 44% amino acid homology with human HSP90α (*11*). In this study, we compared the homodimer-forming characteristics of two HSP90 family-member proteins, barley GRP94 and human HSP90α, and showed that GRP94 exists in a dimeric form, as does HSP90α. The above finding prompted us to test, by use of a coexpression

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Abbreviations: GRP94, the 94-kDa glucose-regulated protein; GST, glutathione *S*-transferase; HSP90α and HSP90β, the α and β isoforms, respectively, of the 90-kDa heat-shock protein; H_6, a histidine-hexamer tag; DSS, disuccinimidyl suberate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

system, the possibility that these two HSP90 family proteins are capable of forming a heterodimeric complex.

MATERIALS AND METHODS

Materials—The histidine-hexamer-tagged expression vector (pQE9-11), pREP4, and Ni-NTA agarose were purchased from Qiagen. The glutathione *S*-transferase (GST)-fusion expression vectors (pGEX-2T and pGEX-4T-1) and glutathione-Sepharose 4B were purchased from Pharmacia Biotech. Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs. Thrombin (108 units/mg protein), *N*-tosyl-L-phenylalanine chloromethyl ketone, and N^{α}-tosyl-L-lysine chloromethyl ketone were purchased from Sigma. All other reagents were of analytical grade.

Expression Vectors Encoding GRP94 cDNA—The cDNA encoding the full length of barley (*Hordeum vulgare* L.) GRP94 (*11*) was cloned into an *Eco*RI site of pBluescript SK(−) (designated pBluescript-GRP94). The expression vector expressing amino acids 69-809 of barley GRP94 with a histidine-hexamer tag at the N-terminus was constructed as follows: pBluescript-GRP94 was double-cut with *Xho*I and *Pst*I, and the fragment encoding amino acids 69-809 was purified by agarose gel electrophoresis. The purified fragment was inserted into a *Sal*I/*Pst*I site of pQE10. For preparation of pQE-GRP94 484-809, pBluescript-GRP94 was double-cut with *Bam*HI and *Pst*I, and the fragment encoding amino acids 484-809 was obtained by agarose gel electrophoresis and then inserted into a corresponding site of pQE10. For pGST-GRP94 426-809, pBluescript GRP94 was cut with *Eco*RI and *Pst*I, and the fragment encoding amino acids 426-809 was purified by agarose gel electrophoresis. The purified fragment was inserted into a corresponding site of pGEX-4T-1.

Expression Vectors Encoding HSP90α cDNA—The cDNA encoding the full-length form of human HSP90α was generously provided by Dr. K. Yokoyama (Riken Life Science Center) (*12*). The expression vectors expressing the full length (amino acids 1-732) and amino acids 533-732 of HSP90α fused to GST (designated pGST-HSP90α and pGST-HSP90α 533-732, respectively) were described earlier (*8*). For preparation of pQE-HSP90α, pGST-HSP90α was double-cut with *Bam*HI and *Pst*I, and a fragment encoding the full length of HSP90α was purified by agarose gel electrophoresis. The purified fragment was then inserted into a corresponding site of pQE9. For pQE-HSP90α 533-732, pQE11 was cut with *Bam*HI and the sticky ends were filled-in with Klenow fragment. The vector was further cut with *Pst*I. pGST-HSP90α was double-cut with *Pvu*II and *Pst*I, and purified by agarose gel electrophoresis. Finally, the *Pvu*II-*Pst*I fragment of HSP90α was ligated with the vector.

Transformation of E. coli Y1090—The *E. coli* Y1090 transformed with pGST-GRP94 426-809 or pGST-HSP90α 533-732 was selected on LB agar containing 50 μg/ml ampicillin. The *E. coli* Y1090 harboring the plasmid pREP4 was transformed with pQE series plasmids (pQE-GRP94 69-809, pQE-GRP94 484-809, pQE-HSP90α, or pQE-HSP90α 533-732), and was selected on LB agar containing 50 μg/ml of each of ampicillin and kanamycin.

Coexpression System—For construction of pGST-GRP94 426-809^{tet} and pGST-HSP90α^{tet}, pBR322 was double-cut with *Eco*RI and *Ava*I, and the staggered ends were filled-in with Klenow fragment. The excised 1.4-kb tet^r gene cassette was purified by agarose gel electrophoresis and then inserted into a blunt-ended and dephosphorylated *Pst*I site located in amp^r gene of pGST-GRP94 426-809 or pGST-HSP90α. Due to the insertion of the tet^r gene, the amp^r gene was disrupted (see Fig. 5a). After transformation of *E. coli* Y1090 with the plasmid, a clone resistant to tetracycline was selected on LB plates containing 100 μg/ml tetracycline. Subsequently, *E. coli* Y1090 harboring pGST-GRP94 426-809^{tet} was transformed with pQE-GRP94 484-809. *E. coli* Y1090 harboring pGST-HSP90α^{tet} was transformed with pQE-GRP94 484-809 or pQE-HSP90α 533-732. The clones resistant to both ampicillin and tetracycline were selected. The plasmid pREP4 encoding the $lacI^q$ gene was not needed in this system, because pGST vectors carry the gene (see Fig. 5a).

Expression and Purification of Recombinant Proteins—The expression of the recombinant proteins encoded by pQE series vectors was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside as described previously (*13*). The bacterial pellet corresponding to a 500-ml culture was suspended in 20 ml of 50 mM sodium phosphate (pH 8.0) and 0.3 M NaCl (Buffer A) containing 10 mM imidazole. Protease inhibitors (20 μg/ml of each of leupeptin, antipain, soybean trypsin inhibitor, and pepstatin and 40 μg/ml of each of tosyl-L-lysine chloromethyl ketone and N^{α}-tosyl-L-phenylalanine chloromethyl ketone) were also included. The cells were lysed with 0.5 mg/ml lysozyme at 0°C for 30 min followed by three cycles of sonication for 30 s at 30-s intervals. The lysate was centrifuged at 20,000× *g* for 20 min at 0°C, and the supernatant was adsorbed on an Ni-NTA agarose column (1.5×3 cm) preequilibrated with Buffer A containing 10 mM imidazole. After extensive washing with Buffer A containing 20 mM imidazole, bound proteins were eluted with 250 mM imidazole (pH 7). The GST-fused proteins encoded by pGST-GRP94 426-809 and pGST-HSP90α 533-732 were expressed and purified as described previously (*14*).

When recombinant proteins were expressed by the coexpression system, the proteins were purified as described above except that the Ni-NTA agarose gel was washed with Buffer A containing 10 mM imidazole instead of 20 mM. The samples (approx. 10 ml) eluted with 250 mM imidazole (pH 7) were subsequently applied to glutathione Sepharose 4B (0.5×3 cm) preequilibrated with phosphate-buffered saline (0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl, and 0.02% KCl; pH 7.3). After extensive washing with the same buffer, the bound proteins were finally eluted with 25 mM Tris-HCl (pH 7.8) containing 10 mM glutathione.

Polyacrylamide Gel Electrophoresis (PAGE)—Proteins were subjected to PAGE under denaturing conditions (*15*). Polyacrylamide gels of 12.5-15% (w/v) were employed unless otherwise stated. Low-molecular-weight markers, CNBr-treated myoglobin (Pharmacia Biotech), and rainbow markers (Amersham) were used as standards.

To characterize the molecular configuration of expressed proteins, we analyzed the proteins by PAGE under nondenaturing conditions (*16*) as described previously (*17*). Ovalbumin (45 kDa), bovine serum albumin (monomer, 66 kDa; dimer, 132 kDa; trimer, 198 kDa), and horse catalase (240 kDa) were used as markers. Polyacrylamide gels of

7.5% (w/v) were employed for native PAGE. Proteins were stained with Coomassie Brilliant Blue R250.

Crosslinking—Purified proteins (20 μg) were diluted to 0.2 mg/ml with phosphate-buffered saline (pH 7.3). The crosslinking was performed with 1 mM disuccinimidyl suberate (Pierce) at 30°C. After a 1-h incubation, the reaction was stopped by incubation with 0.1 M glycine (pH 7) for 10 min. The sample was then precipitated with 15% (w/v) cold trichloroacetic acid. The precipitate was solubilized and denatured with SDS-sample buffer (*15*); and then an aliquot (5 μg) was analyzed by SDS/PAGE. Ovalbumin (45 kDa) and soybean trypsin inhibitor (21.5 kDa), which are present as monomers, were used as negative controls.

N-Terminal Sequence Analysis—The purified proteins were incubated with thrombin at 30°C for 10 h. Following denaturation, electrophoresis, and blotting onto a polyvinylidene difluoride membrane (Bio-Rad), protein bands were stained and subjected to N-terminal sequence analysis as described previously (*8*).

RESULTS

Homodimerization of GRP94 and HSP90α—The barley GRP94 is composed of 809 amino acid residues, and the first 20 amino acids seem to be processed in the mature form (*11*). Thus, the mature form of barley GRP94 is 57 amino acids longer than human HSP90α. This difference is principally due to the extra amino acids present at the N-terminus of barley GRP94. In this study, we first expressed a nearly full-length form of barley GRP94 (amino acids 69-809) and the full-length form (amino acids 1-732) of human HSP90α in *E. coli* with a histidine-hexamer tag at the N-terminus. The expressed proteins, designated H_6GRP94 69-809 and H_6HSP90α, respectively, were purified to homogeneity by affinity chromatography on Ni-NTA agarose. Their molecular masses were 91 kDa for H_6GRP94 69-809 and 90 kDa for H_6HSP90α (Fig. 1a). The small difference in position between the two proteins reflected the difference in their calculated molecular masses. Native PAGE analysis revealed that H_6GRP94 69-809 and H_6HSP90α migrated predominantly to positions corresponding to 198 and 210 kDa, respectively (Fig. 1b). Unexpectedly, the positions were the reverse of those observed on SDS/PAGE, although the difference was not significant. Comparison of the molecular masses determined by the two kinds of PAGE indicated that GRP94 and HSP90α both existed in the dimeric form. In the case of H_6HSP90α, additional bands with higher molecular masses were found (arrowheads in Fig. 1b). These species may correspond to oligomeric forms.

It has been demonstrated that the C-terminal 200 amino acids of HSP90α are sufficient for the dimerization (*8*). Thus, to compare the modes of dimerization of GRP94 and HSP90α, we expressed the C-terminal region of GRP94, H_6GRP94 484-809, which consists of the C-terminal 326 amino acid residues of the protein. On SDS/PAGE, H_6GRP94 484-809 migrated to the position of 42 kDa (Fig. 2a, lane 1). Under the native PAGE conditions, the same protein migrated at 140 kDa (Fig. 2b, lane 1), which indicates trimer formation of H_6GRP94 484-809. Similarly, the C-terminal 200 amino acids of HSP90α, appearing at 26 kDa on SDS/PAGE (lane 2), migrated to the position of 80 kDa, again indicating a trimeric structure. However, the latter finding is apparently contradictory to our recent report demonstrating a dimeric structure of HSP90α 533-732 (*8*). We (*17*) and others (*18, 19*) previously noted that migration of proteins on native PAGE is greatly affected by the net charge. Since the tagged peptide, Met-Arg-Gly-Ser-His_6, encoded by the vector is highly basic, it may cause the retardation on native PAGE. Thus, we suppose that the proteins existed as dimers, but these apparently migrated at the position of trimers on native SDS/PAGE.

To investigate further the native structure of the expressed proteins, they were crosslinked with disuccinimidyl suberate and analyzed by SDS/PAGE. Following the crosslinking reaction of H_6HSP90 533-732, a band at 60 kDa was found (Fig. 3, lane 4). The 60-kDa species should correspond to a dimer of H_6HSP90α 533-732. Under the same conditions, crosslinking of ovalbumin (45 kDa) and

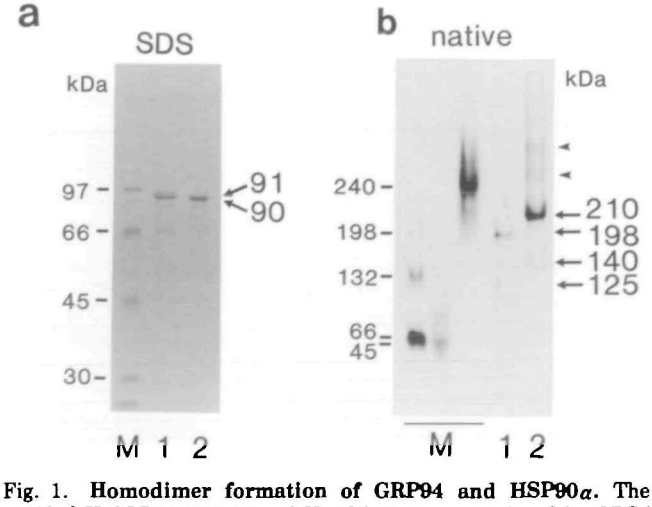


Fig. 1. **Homodimer formation of GRP94 and HSP90α.** The purified H_6GRP94 69-809 and H_6HSP90α were analyzed by SDS/PAGE (a) or native PAGE (b). Three and 6 μg of proteins were loaded on SDS and native gels, respectively. Lane 1, H_6GRP94 69-809; lane 2, H_6HSP90α. Arrowheads indicate oligomeric species. Lane M, molecular-weight markers.

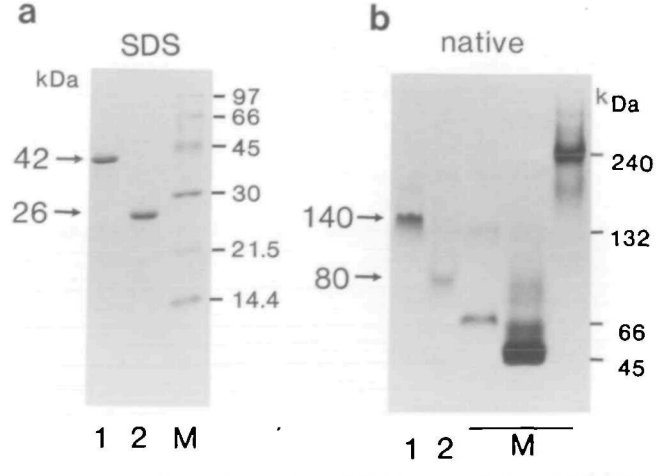


Fig. 2. **Homodimer formation of GRP94 484-809 and HSP90α 533-732.** The purified H_6GRP94 484-809 and H_6HSP90α 533-732 were analyzed by SDS/PAGE (a) or native PAGE (b). Lane 1, H_6GRP94 484-809; lane 2, H_6HSP90α 533-732. Lane M, molecular-weight markers.

soybean trypsin inhibitor (21.5 kDa), which exist as monomers in solution, did not reveal any larger-molecular-mass species (Fig. 3, lanes 5-8). Thus, as we speculated above, H_6HSP90α 533-732 existed as a dimer, but was retarded on native PAGE because of its basic character. Although apparent retardation on native PAGE was not found on the full-length molecules (Fig. 1b), we presume that the effect of the histidine tag is negligible on large-molecular-mass proteins.

In the case of H_6GRP94 484-809, an 80-kDa band and a broad band larger than 100 kDa were observed (Fig. 3, lane 2). Electrophoresis of the same sample at a lower polyacrylamide concentration revealed the 80-kDa species and a species larger than 200 kDa (lane 10). A faint band was also found at around 160 kDa. The 80- and 160-kDa species should correspond to a dimer and a tetramer, respectively. The largest band may correspond to a hexamer. Note that a band corresponding to a trimer (126 kDa) was not detected. This finding is somewhat contradictory to the result of native PAGE, in which the oligomeric forms of H_6GRP94 484-809 were not detected (Fig. 2b, lane 1). Nevertheless, it should be noted that the C-terminal 326 amino acids of GRP94 were sufficient for dimerization to occur.

Limited Proteolysis of the C-Terminal Region—The site of HSP90 susceptible to proteolysis is located at the C-terminal region of the molecule, and the deletion of the C-terminal region causes the dissociation of the HSP90 dimer (*7, 8*). So, we compared the proteolytic patterns of GRP94 and HSP90α. We used GST fusion-proteins for this experiment because the cleavage sites had been unambiguously determined with GST-HSP90α 533-732 (*8*).

Proteolysis of GST-HSP90α 533-732 with thrombin generated the 26-kDa HSP90α 533-732 and the 27-kDa GST (Fig. 4a, lanes 9 and 10). In addition, two smaller fragments of 13 and 16 kDa were detected. As reported earlier (*8*), these 16- and 13-kDa fragments are derived from the C-terminus and the adjacent N-terminal region of HSP90α 533-732, respectively, and are associated with each other.

Principally, the cleavage of the thrombin recognition site of GST-GRP94 484-809 was expected to produce 27- and 47-kDa species derived from GST and GRP94 426-809, respectively. However, several extra bands, *i.e.*, 16-, 35-, and 64-kDa species, were observed after thrombin treatment (Fig. 4a, lanes 2-7). The 16-kDa band was faint but was reproducibly found at higher thrombin concentrations (lanes 6 and 7). In some experiments, the 16-kDa species split into a doublet (lane 11, designated 16A and 16B). Note that a 16-kDa species was also found for GST-HSP90α 533-732. Although a faint 13-kDa band appeared at the highest thrombin concentration (lane 7), we could not further characterize it because of the limited amount formed.

To address the origin of these proteolytic fragments, their N-terminal sequences were determined (Table I). The 27- and 64-kDa species had the N-terminal sequence of GST. The 35- and 47-kDa species had the sequence corresponding to the GRP94 moiety of the fusion protein. On the other hand, the 16-kDa species was produced through the cleavage of the intramolecular sequence of GRP94 426-809. Hence, the origin of the proteolytic fragments was considered to be as shown in Fig. 4b, which clearly demonstrates the localization of the region susceptible to thrombin at the position one-third from the C-terminus of GRP94 426-809. That is, the specific cleavage of the Arg710-His711 and/or Arg735-Leu736 bond in addition to the cleavage at the authentic site accounts for the generation of the proteolytic fragments (Fig. 4, b and c).

Coexpression of Two HSP90-Family Proteins—Interestingly, a minor population of HSP90α and HSP90β purified from mouse lymphoma cells exists as a heterodimer (*5*). Thus, considering the structural similarity of GRP94 and HSP90α described above, we tested the possibility that GRP94 and HSP90α are capable of forming a heterodimeric complex. For this purpose, we developed a novel coexpression system (Fig. 5a). By introducing a *tet*^{r} gene cassette into an *amp*^{r} gene, pGST-GRP94 426-809, and pGST-HSP90α were converted to *tet*^{r}/*amp*^{s} plasmids, pGST-GRP94 426-809^{tet} and pGST-HSP90α^{tet}, respectively. In the growth phase, the expression of recombinant proteins was suppressed by the *lac* repressor carried on the *lacI*^{q} gene of pGST vectors. Then, the expression was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside. If the two proteins expressed were associated in the bacterial cytoplasm, they should be retained on both Ni-NTA agarose and glutathione Sepharose affinity columns.

As a control experiment, GRP94 426-809 fused to GST and GRP94 484-809 tagged with a histidine hexamer were coexpressed in *E. coli*. The expressed proteins were first subjected to chromatography on Ni-NTA agarose, which

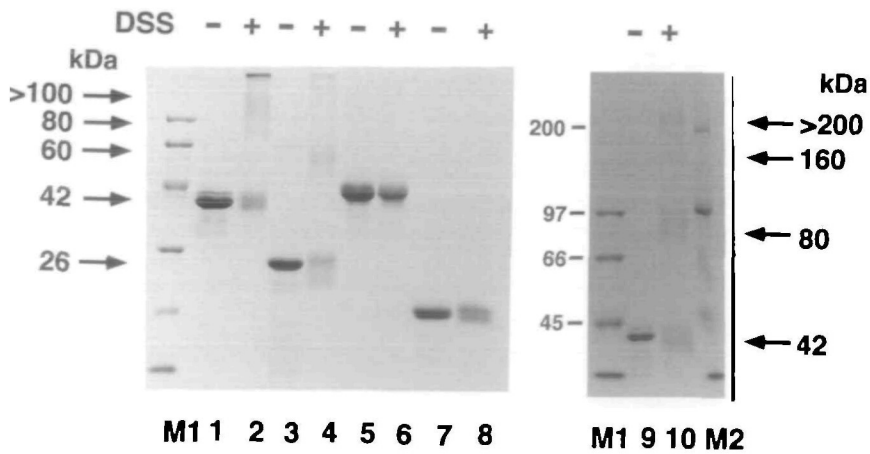
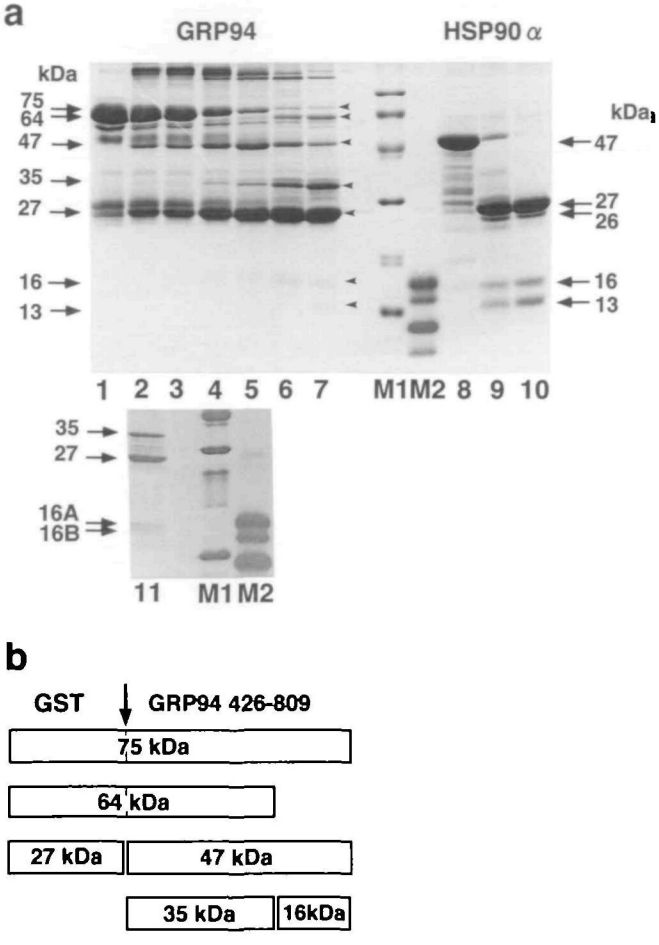


Fig. 3. **Crosslinking analysis of dimeric species.** The purified H_6GRP94 484-809 and H_6HSP90α 533-732 (20 μg/0.1 ml) were crosslinked as described in "MATERIALS AND METHODS." The samples (5 μg) were analyzed by SDS/PAGE. H_6GRP94 484-809 (lanes 1, 2, 9, and 10), H_6HSP90α 533-732 (lanes 3 and 4), ovalbumin (lanes 5 and 6), and soybean trypsin inhibitor (lanes 7 and 8), were incubated without or with disuccinimidyl suberate (DSS), and then analyzed by SDS/PAGE on a 12.5% (lanes 1-8) or 7.5% (lanes 9 and 10) polyacrylamide gel. M1, low-molecular-weight markers; M2, rainbow markers.

trapped the protein with a histidine-hexamer tag. Proteins eluted with 250 mM imidazole (pH 7) were immediately poured onto a glutathione-Sepharose column, which trapped GST-fusion proteins. A considerable amount of 75-kDa GST-GRP94 426-809 as well as 42-kDa H_6GRP94 484-809 bound to the first-step affinity column (Fig. 5b, lane 2); and finally, substantial amounts of the two proteins were recovered from the second-step affinity chromatography (lane 4). Similarly, the affinity chromatography demonstrated the association of GST-HSP90α and H_6-HSP90α 533-732 (Fig. 5c). The interaction of the two proteins was further confirmed by two-dimensional PAGE (Fig. 5e): in the first-dimensional native PAGE, the purified sample migrated broadly with molecular masses larger than 198 kDa. The second-dimensional SDS/PAGE showed a band composed of both the 26-kDa H_6HSP90α 533-732



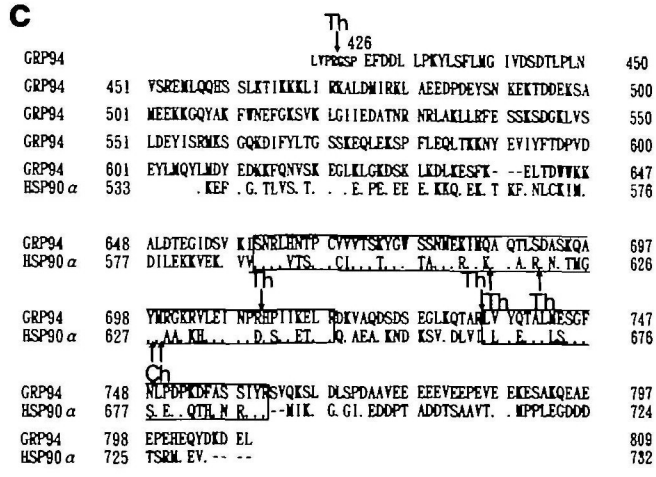


Fig. 4. **Thrombin cleavage of GST-GRP94 426-809.** (a) GST-GRP94 426-809 (lanes 1-7 and 11) or GST-HSP90α 533-732 (lanes 8-10) (100 μg for each lane sample) was incubated without or with thrombin at 30°C for 10 h. An aliquot (25 μg/lane) was separated by SDS/PAGE. The doses of thrombin are; none (lanes 1 and 8), 0.03 μg (lane 2), 0.1 μg (lane 3), 0.3 μg (lanes 4 and 9), 0.9 μg (lanes 5 and 10), 2.7 μg (lane 6), and 8.1 μg (lane 7). Lane M1, low-molecular-weight markers; lane M2, CNBr-treated myoglobin (17 kDa, 14.4 kDa, 10.7/8.2 kDa, and 6.2 kDa from the top of the gel). The molecular masses of the fragments derived from GST-GRP94 426-809 and GST-HSP90α 533-732 are indicated on the left and right, respectively. In three of five separate experiments, a 16-kDa doublet was observed (lane 11, designated 16A and 16B). The remaining protein samples of lanes 6 and 11 (75 μg each) were subjected to N-terminal sequencing analysis (see Table I). (b) The origin of the fragments. An arrow indicates the authentic thrombin cleavage site encoded by the vector. (c) The proteolytic cleavage sites of GRP94 426-809 and HSP90α 533-732 (*8*) are compared. Amino acid sequences are represented in single letters. Small capital letters indicate amino acids derived from GST. Amino acids of HSP90α identical to those of GRP94 are indicated as dots. Hyphens are inserted to maximize matching. Two amino acid stretches conserved between the two proteins are boxed. Th, thrombin cleavage site; Ch, chymotryptic cleavage site.

TABLE I. **Amino acid sequences of the proteolytic fragments of GST-GRP94 426-809.** The amino acids derived from GRP94 426-809 are indicated in bold letters with their amino acid numbers in the last column. The sequence "MSPILG" corresponds to the N-terminal sequence of GST.

Peptide (kDa)	Detected amino acids	Corresponding sequence
16A	LVYQTALMES	736 **LVYQTALMES**
16B	HPI I KELRDKVAQDSDS	711 **HPI I KELRDKVAQDSDS**
27	MSPI L	**MSPI L**
	SPI LG	**SPI LG**
35	GSPEFDDLL	426 **GSPEFDDLL**
47	GSPEFDDLLP	426 **GSPEFDDLLP**
64	MSPI L	**MSPI L**
	SPI LG	**SPI LG**

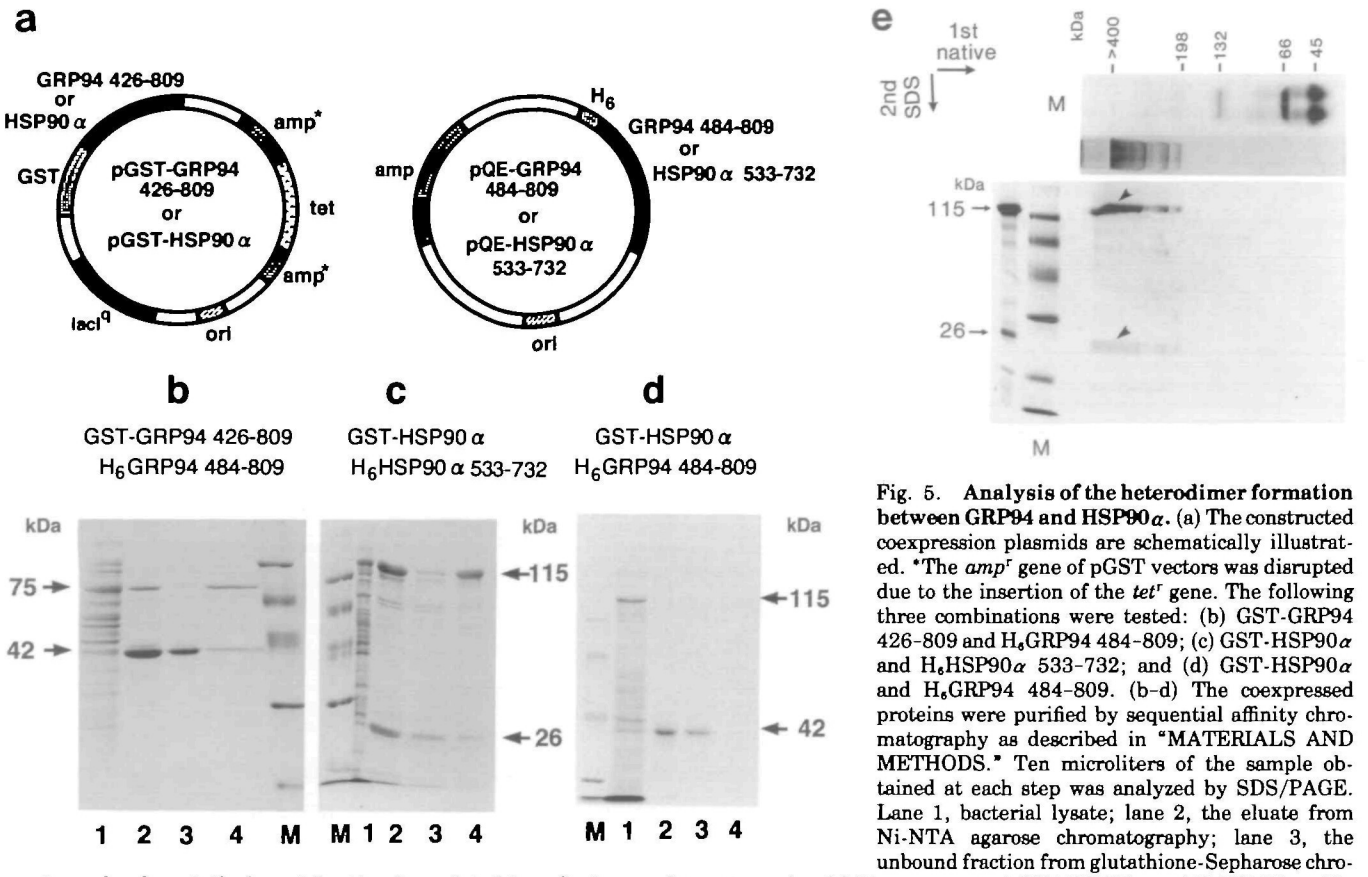


Fig. 5. **Analysis of the heterodimer formation between GRP94 and HSP90α.** (a) The constructed coexpression plasmids are schematically illustrated. "The *amp*^{r} gene of pGST vectors was disrupted due to the insertion of the *tet*^{r} gene. The following three combinations were tested: (b) GST-GRP94 426-809 and H_6GRP94 484-809; (c) GST-HSP90α and H_6HSP90α 533-732; and (d) GST-HSP90α and H_6GRP94 484-809. (b-d) The coexpressed proteins were purified by sequential affinity chromatography as described in "MATERIALS AND METHODS." Ten microliters of the sample obtained at each step was analyzed by SDS/PAGE. Lane 1, bacterial lysate; lane 2, the eluate from Ni-NTA agarose chromatography; lane 3, the unbound fraction from glutathione-Sepharose chromatography; lane 4, the bound fraction from glutathione-Sepharose chromatography. (e) The coexpressed GST-HSP90α and H_6HSP90α 533-732 (lane 4 of panel c) were separated by native PAGE as the first dimension and SDS/PAGE as the second dimension. GST-HSP90α (115 kDa) and H_6HSP90α 533-732 (26 kDa) are indicated by arrowheads.

and the 115-kDa GST-HSP90α. Hence, this coexpression system can be used to examine whether or not GRP94 and HSP90α form a heterodimeric complex.

We then applied the technique to the coexpressed forms of H_6GRP94 484-809 and GST-HSP90α. Although both H_6GRP94 484-809 and GST-HSP90α were major constituents of the lysate (Fig. 5d, lane 1), only H_6GRP94 484-809 was purified at the first-step chromatography (lane 2). At the second step, essentially all the H_6GRP94 484-809 passed through glutathione Sepharose (lane 3), and no protein was recovered in the final eluate (lane 4). Therefore, we conclude that GRP94 484-809 did not interact with HSP90α even when they were coexpressed in the same *E. coli* cell.

DISCUSSION

This study demonstrated the dimer-forming ability of barley GRP94. GRP94 as well as HSP90α exists as a dimer through self-association of the C-terminal region. Although the dimer-forming ability is limited for HSP90β, another HSP90-family member, it still tends to form a dimer to some extent (*5*); the impediment to dimerization is at least 16 amino acid substitutions between the α and β isoforms in the C-terminal region (amino acids 561-685) (*8*). Therefore, we propose that the dimer formation through the interaction of the C-terminal region is a general property of the HSP90-family member proteins.

We found that a minor population of HSP90α existed in oligomers (Fig. 1b, lane 2). Recent studies have demonstrated the oligomerization of HSP90 under certain conditions, such as in the presence of divalent cations (*20*) and at elevated temperatures (*21*). Furthermore, Bresnick *et al.* (*22*) reported that oligomers larger than the dimer of HSP90 are associated with the glucocorticoid receptor purified by immuno-affinity chromatography under mild conditions. We found that the state of HSP90 varied depending on the measuring conditions (T. Nemoto, unpublished observation): Namely, HSP90 oligomers were detected by gel chromatography, but HSP90α was predominantly present as a dimer and HSP90β mainly as a mixture of a dimer and a monomer when analyzed by native PAGE. The crosslinking experiment in this study also suggested the presence of oligomeric species of GRP94 484-809, although the native PAGE analysis did not reveal those forms. The interaction for the oligomer formation seems to be relatively weak compared to the interaction for the dimer formation. Further studies are needed to examine the oligomerization of GRP94 *in vitro* and *in vivo*.

Amino acid comparison indicates that one of the thrombin cleavage sites of GRP94, Arg710-His711, is close to those of HSP90α (see Fig. 4c). On the other hand, the second site (Arg735-Leu736) is separated by 25 amino acids from the nearest proteolytic site of HSP90α, but this is still near when we take into account the expressed length (384 amino acids). Accordingly, we conclude that the

proteolytic susceptibility of the C-terminal region is a general characteristic of the HSP90-family proteins. This finding strongly suggests that a dimeric structure similar to that of HSP90 is maintained for GRP94.

By use of the coexpression system developed herein, we examined whether or not the two HSP90-family proteins form a heterodimeric complex. Even if such complex formation is possible under *in vitro* conditions, it would be physiologically meaningless because the two proteins are localized in distinct subcellular compartments, but we performed this experiment to clarify the structural requirements of dimer formation in GRP94 and HSP90α. Control experiments demonstrated that GRP94 426-809 and HSP90α formed complexes with GRP94 484-809 and HSP90α 533-732, respectively. However, we failed to detect an interaction between GRP94 and HSP90α. Therefore, the fine structure of the homodimeric interaction of GRP94 seems to be different from that of HSP90α, although the mode of dimer formation is basically identical. The finding that one of the thrombin cleavage sites of GRP94 (Arg735-Leu736) is somewhat separated from those of HSP90α may be related to the difference in dimeric configuration between the two proteins.

Several systems expressing multiple proteins in a single *E. coli* cell have been developed. Several investigators have used a single vector which carries multiple genes to be expressed (*23, 24*), and others have used multiple and compatible expression vectors each of which encodes one protein to be expressed (*25*). In this study, we employed the latter system for the following reasons. Firstly, the second plasmid can be constructed simply by the insertion of the *tet*^{r} fragment into the ampicillin-resistant GST-fusion plasmid; Secondly, both of the expressed proteins that contain either GST or the histidine-hexamer tag are readily purified by affinity chromatography, and thus, the associated proteins are purified to homogeneity by sequential affinity chromatography (Fig. 5). The coexpression system developed herein should be generally applicable to the analysis of the homo- and hetero-oligomeric interactions of two proteins. It is important to note that the expressed proteins retain native conformations, as they are purified under nondenaturing conditions.

The amino acid sequence of barley GRP94 is 44% homologous to those of human HSP90α and HSP90β, and the C-terminal region of GRP94 (amino acids 607-809) is 37% homologous to the C-terminal 200 amino acids of HSP90α. Among the C-terminal region, two stretches, *i.e.*, amino acids 660-718 and 736-761 of GRP94, are 59 and 58%, respectively, conserved in relation to the corresponding sequences of HSP90α (indicated as boxes in Fig. 4c). These regions may be important for the dimeric interaction of the proteins in this family. On the other hand, the amino acids around the C-terminus are not conserved between GRP94 and HSP90α. This accords with the previous finding that replacement of the C-terminal 35 amino acids of HSP90α with those of HSP90β did not affect the dimer-forming ability (*8*).

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