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Selective Deletions in the 90 kDa Heat Shock Protein (hsp90) Impede Hetero-oligomeric Complex Formation with the Glucocorticosteroid Receptor (GR) or Hormone Binding by GR

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We have developed an *in vivo* system using coexpression of human glucocorticosteroid receptor (hGR) and chick hsp90α (chsp90) in recombinant virus-infected Sf9 cells to study the formation of hetero-oligomeric complexes. We detected, in the cytosol, hGR complexes containing chsp90 as shown by the displacement of the [^{3}H]triamcinolone acetonide bound hGR "8S" peak on glycerol/sucrose gradients by specific antibodies directed against chsp90 (BF4 and D7α). We took advantage of this system and of the immunoadsorption of hGR containing complexes with anti-hGR antibody to analyze the effect of deletions introduced into the hsp90 molecule on the formation of complexes with the hGR. Deletion of the hydrophilic region "A", between amino-acids 221 and 290, abolished the formation of hGR/chsp90 complexes. Deletion of the hydrophilic region "B" (between amino-acids 530 and 581) or deletion of a leucine repeat region "Z" in the middle of the molecule (amino-acids 392 to 419) still allowed formation of hetero-oligomeric complexes detected by immunoadsorption but the hGR complexes formed with mutated chsp90s were devoid of steroid binding properties. These results are consistent with (1) the possible involvement of the "A" region in the interaction of hsp90 with steroid receptors and (2) a role of B and Z regions in the hsp90 structure for maintaining the steroid binding property of the hGR.

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INTRODUCTION

The 90 kDa heat shock proteins (hsp90) belong to a class of highly conserved proteins which are crucial for cell viability [1, 2]. It is believed that hsp90s associate with various cellular proteins such as steroid hormone receptors and possibly maintain these proteins in a nonfunctional state. Steroid receptors, in the absence of hormone, form hetero-oligomeric complexes containing a hsp90 dimer and these are dissociated by hormone, allowing them to acquire DNA binding capacity and transcriptional activity according to the corresponding activation process [3–6]. For the glucocorticosteroid receptor, where high affinity steroid binding occurs only in the hetero-oligomeric form, hsp90 is involved in maintaining the appropriate conformation of the ligand binding domain (LBD) [7–9]. Analysis of mutated steroid receptors have suggested that the LBD interacts directly with hsp90 [10–13] but no reciprocal analysis of functional regions of hsp90 has yet been undertaken. Here, we describe the formation of hetero-oligomeric complexes from exogenous chick hsp90α (chsp90) coexpressed with the human glucocorticosteroid receptor (hGR) in Sf9 cells infected with recombinant baculovirus. We investigated the role of three regions of the hsp90 by analyzing the effects of corresponding deletions in the molecule on the interaction with the hGR. Two derivatives were deleted in highly charged regions which are potential candidates for interaction with steroid receptors [14, 15], and a third was devoid of a leucine heptad repeat which could be involved in protein–protein contacts.

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EXPERIMENTAL

Generation of recombinant baculoviruses

Chsp90 cDNA [14] was inserted into the BamHI site of the baculovirus transfer vector PVL941 [16] after filling in all protruding ends, deletions of the A region (amino-acids 221–290) and B region (amino-acids 530–581) or Z region (amino-acids 392–419) were obtained by oligonucleotide directed mutagenesis (N.B., Marc Lombes and E.E.B., in preparation, and Beatrice Chambraud, unpublished data). *In vivo* homologous recombination took place after cotransfection of vector plasmid and of baculovirus type DNA (Autographa Californica Nuclear polyhedrosis virus or AcMNPV) in *Spodoptera frugiperda* (Sf9) cells accordingly to the calcium phosphate precipitation method [16]. Screening of recombinants was performed by microscopic observation of negative occlusion plaques, followed by serial plaque purification [16].

The hGR sequence between KpnI and XhoI of pRShGRα [17] was introduced into the NheI site of the PBlueBac vector (Invitrogen) after filling in protruding ends. hGR sequence-containing constructs were selected by hybridization with hGR fragments. After cotransfection with DNA, recombinant viruses were identified by their β-galactosidase activity, detected by development of a blue color when 150 μg/ml bluogal (BRL) was introduced in a 1% agar layer, as described by Vialard *et al.* [18], and then purified by three serial plaque rounds.

In all cases, expressed proteins were analyzed for their molecular weight and the hGR for its ability to bind steroid.

Sf9 culture and infection

Sf9 cells were grown in spinner flasks containing TNM-FH medium (Sigma) containing 10% steroid-depleted fetal calf serum [19]. Stock virus suspensions were obtained by infecting 1.8×10^7 Sf9 cells with 0.1 ml of either the 1 ml virus inoculum (after the third plaque round) or 0.1 ml of a previous viral stock (multiplicity of infection 1 to 10) in 4 ml suspension medium for 2 h before introducing the mixture in a preconditioned 150 cm^2 flask containing 21 ml of complete fresh medium to allow cells to attach. After 5 days, we checked that all cells were infected and produced viruses, and obtained a stock virus suspension after pelleting detached cells for 10 min at 3000 rpm. Coinfection was performed by adding 3 ml of chsp90-expressing virus suspension to 6×10^7 Sf9 cells in 6 ml of TNM-FH medium containing 10% steroid-depleted serum in a 50 ml polypropylene tube and 2 h later 3 ml of the hGR-expressing virus suspension. After a further 2 h, the cells were added to 60 ml of TNM-FH medium with 10% steroid-depleted serum in a spinner flask. They were harvested at different times after infection (usually 36 h).

Labeling and preparation of cellular extracts

For the metabolic labeling of Sf9 cells, 6×10^7 cells were carefully washed in Grace's medium without methionine (Met) 24 h after coinfection, then cultured for 6 h in Grace's medium containing 1 mCi of [^{35}S]Met.

Cytosols from Met-labeled or unlabeled cells were prepared as described previously [10] in GTED buffer containing 15 mM sodium molybdate except that homogenization was performed in an all-glass motorized homogenizer. Steroid binding capacities were determined by incubating cytosols with 200 nM tritiated triamcinolone acetonide ([^{3}H]TA) for 2 h and treating them according to the dextran-coated charcoal technique [11].

Sedimentation analysis on sucrose gradients

Aliquots were layered on 5–25% sucrose gradients (5 ml) prepared in TED buffer (20 mM Tris–Cl, 1 mM EDTA, 1 mM dithioerythritol, pH 7.4 at 4°C) containing 10 or 13% v/v glycerol. A 65,000 rpm centrifugation was performed for 2 h in a VTI 65.2 rotor in the presence of internal standards (glucose oxidase, 7.9S and horseradish peroxidase, 3.6S). Fractions (3 drops) were collected and counted, or used for Western blots. Aliquots were taken to measure peroxidase and glucose-oxidase activities as described previously [11].

Immunodetection of the hGR and hsp90

BF4 and D7α are monoclonal antibodies raised against chsp90 and are specific for that species [20, 21]. AC19 is an anti-hsp90 antibody that only works in Western blots, but recognizes hsp90 from various species including insect hsp90. The anti-hGR is a rabbit polyclonal antibody raised against the hGR peptide containing amino-acids 149 to 168, it can displace both 8 and 4S hGR towards high molecular weight fractions on sucrose/glycerol gradient and interacts with the SDS-denatured form of the hGR on Western blot. It also can be used for immunohistological studies.

Western blots were performed as described previously [10]. Briefly, saturation with nonfat dry milk (10%) was followed by incubation with either anti-chsp90 antibodies (BF4 or AC19) or anti-hGR antibody. Bound antibody on Western blot was revealed by the immunoperoxidase technique using the appropriate secondary biotinylated antibody, avidin–biotin peroxidase complex (Vectastain ABC) and a chemiluminescent substrate (Amersham, ECL system).

Immunoadsorption of hGR-containing complexes was carried out on an anti-hGR protein-A-Sepharose resin prepared as described by Hutchison *et al.* [22]. Resins containing 4–5 mg/ml of either purified anti-hGR antibody or a control-IgG were cross-linked as described by Schneider *et al.* [23]. In short, cytosols were depleted of free hsp90 and nonspecific contaminants by two successive adsorption runs on control IgG resin. Supernatants of 50 to 600 μl of cytosols were immunoadsorbed on 0.1 ml of control IgG or specific

resin. After washing, adsorbed proteins were eluted by 50 mM Tris–Cl, pH 10.9 (three times 0.4 ml for 30 min on a rotating wheel), adjusted to 0.5 M NaCl and precipitated by 80% acetone at −20°C, then analyzed by Western blot using the AC19 antibody. When [^{35}S]Met labeled cytosols were used, SDS gels were enhanced with Amplify™ (Amersham), dried and autoradiographed. Quantification of the intensities of the bands was performed using a Bioimage Electrophoresis Analyzer (Millipore).

RESULTS

Coexpression of the hGR and native or deleted chsp90α in Sf9 cells

We studied the expression of various chsp90s and hGR after 24, 36 and 48 h. Steroid binding of the hGR was optimal at 24 h although the receptor protein accumulated until 48 h without increasing its steroid binding capacity, suggesting limiting or regulatory events. At 24 h, the level of chsp90s was inferior to the level of endogenous insect hsp90, assuming that the 90 kDa band stained by Coomassie blue represented mainly insect hsp90 (not shown). Therefore, we chose an infection time >36 h that allowed accumulation of sufficient quantities of chsp90 that could compete efficiently with the endogenous hsp90 to form a complex with the hGR.

Figure 1 shows the results obtained after a 36 h infection period. The hGR is not detectable by SDS–PAGE analysis after protein staining by Coomassie Blue but is easily detected by ECL Western blot using anti-hGR antibody [Fig. 1(a and c)]. Native and deleted chsp90s are visualized both by protein gel and Western blot with antibody AC19 [Fig. 1 (a and b)]. Comparison of the intensities of the hsp90 bands stained by Coomassie blue [Fig. 1(a)] and detected by the Western blot technique illustrates that AC19 is less efficient in detecting insect hsp90 than chsp90 [compare Fig. 1 (a and b), lanes 1 and 5].

The three deletions introduced into chsp90α are illustrated schematically by the diagram in Fig. 2. Region A (amino-acids 221–290) corresponds to the most negatively charged region of the hsp90 molecule. Region B (amino-acids 530–581) is also a charged segment while region Z (amino-acids 392–419) is characterized by a leucine heptad repeat at the centre of the molecule. The migrations of various chsp90s correspond to their expected molecular weights [Fig. 1(a and b)].

Formation of 8S hGR–chsp90 hetero-oligomeric complexes

Sedimentation analysis on sucrose gradients of [^{3}H]TA-labeled cytosol aliquots of coinfected Sf9 cells expressing hGR and polyhedrin (a wild type baculovirus protein), gave the usual hGR profile with a major peak at 8S (Fig. 3, open square symbols), corresponding to the hetero-oligomeric complexes. If incubation with the anti-chsp90 antibodies BF4 or D7α was performed, no displacement of this "8S" peak was observed [Fig. 3 (a and c)], indicating that antibodies did not interact with the insect hsp90.

When Sf9 cells were coinfected with both hGR and wild type chsp90 expression viruses for 36 h, the anti-chsp90 antibodies totally displaced the [^{3}H]TA "8S" peak to the 11S position, indicating that in these circumstances, the ligand-bound receptor interacts preferentially with chsp90 [Fig. 3 (b and d)]. Control ascites or rat IgG gave negative results. That a real competition took place between chsp90 and endogenous hsp90 was assessed by total 8S peak displacement observed after 36 and 48 h cell infection while after 24 h only 40% of peak radioactivity was shifted to 11S (not shown). On the basis of these results, an analysis of the chsp90 subregion functions using mutagenesis techniques was undertaken.

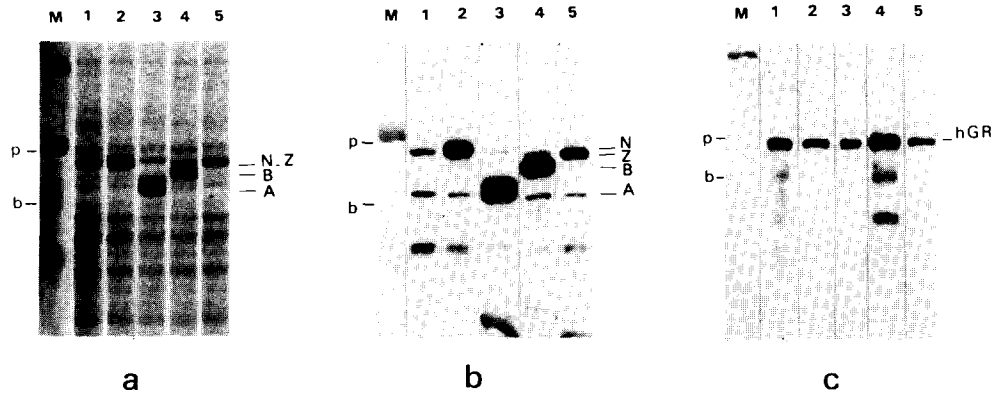

Fig. 1. Coexpression of the GR with various hsp90s in Sf9 cells. Cytosols were prepared as described in Experimental and aliquots were run on 7.5% SDS–PAGE gels: without chsp90 (endogenous insect hsp90, lane 1), with wild-type chsp90 (lane 2), A-deleted chsp90 (lane 3), B-deleted chsp90 (lane 4) or Z-deleted chsp90 (lane 5), Rainbow™ protein molecular weight markers (Amersham): p, phosphorylase *b*, 97.4 kDa and b, bovine serum albumin, 69 kDa. (a) 95 μg of protein of the cytosol from cells expressing GR alone (lane 1) and 50 μg of protein of other cytosols (lanes 2–5), prepared as described in Experimental, were run on SDS gels and stained by Coomassie blue. (b) hsp90s; Western blot of 19 μg (lane 1) and 10 μmg (lanes 2–5) of cytosolic protein using AC19 antibody. (c) hGR; Western blot of 38 μg (lane 1) and 20 μg (lanes 2–5) of cytosolic protein, using anti-hGR polyclonal antibody. N, Z, B, and A indicates the positions of native or wild-type chsp90, Z-, B- and A-deleted chsp90s, respectively.

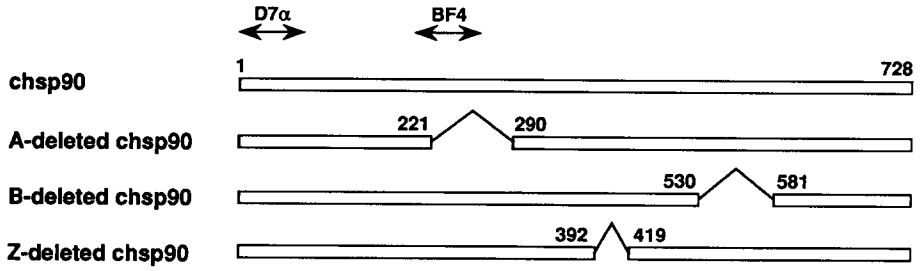

Fig. 2. Schematic representation of chsp90 mutants and epitope map of BF4 and D7α antibodies. Epitope localization of D7α was determined by W. P. Sullivan and D. O. Toft (personal communication and [34]).

Mutated hsp90s cannot form ligand-bound 8S complexes

None of the mutated chsp90s led to the formation of [^{3}H]TA-bound 8S complexes which could be displaced by specific anti-chsp90 antibodies [Fig. 4 (a, b and c)]. We checked that epitopes of the free, not-included in receptor complexes, mutated chsp90s remained accessible to specific anti-chsp90 BF4 and D7α antibodies except BF4 for the A-deleted chsp90 (not shown). Since wild-type chsp90–hGR complexes interact with the same antibodies, complete epitope masking upon hGR interaction with mutated chsp90 is unlikely. Therefore, the lack of interaction with chsp90 antibodies, when deletions are introduced into chsp90, should indicate that ligand-bound hGR complexes were only made with insect hsp90.

Two distinct mechanisms may generate such a result; either deletions abolish the capacity of chsp90 to interact with the hGR, or if interaction still occurs, these deletions impede the ability of chsp90 to maintain a steroid binding conformation of the LBD of the hGR. Therefore, the next step was to analyze the possible formation of complexes between chsp90 and the hGR devoid of steroid binding activity.

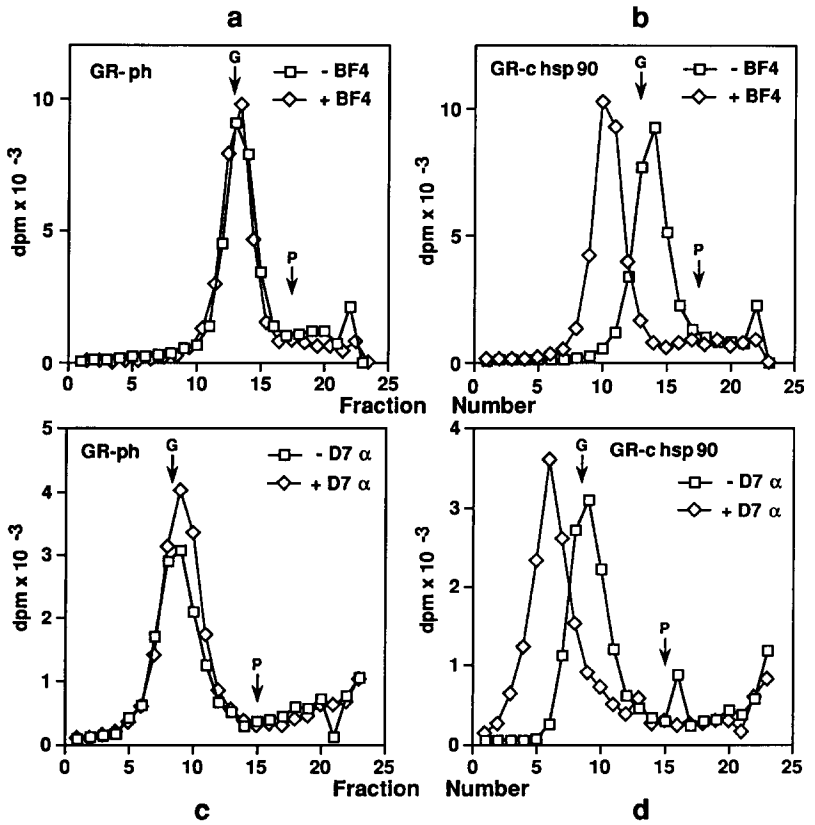

Fig. 3. Sedimentation analysis of [^{3}H]TA-bound hGR coexpressed with or without wild-type chsp90. Effect of D7α and BF4 antibodies. Cytosol aliquots from Sf9 cells coinfected with the GR and polyhedrin (ph) expression viruses (120 μg) (a and c) or wild-type chsp90 (60 μg) (b and d) were diluted in GTED buffer and incubated for 2 h in the absence (□) or presence (◇) of 25 μl of BF4 (a and b) or 5 μl of D7α (c and d) antibodies (ascites fluids). Centrifugation on sucrose/glycerol gradients (13% v/v glycerol in a and b and 10% v/v glycerol in c and d) and sedimentation analyses were performed as described in Experimental.

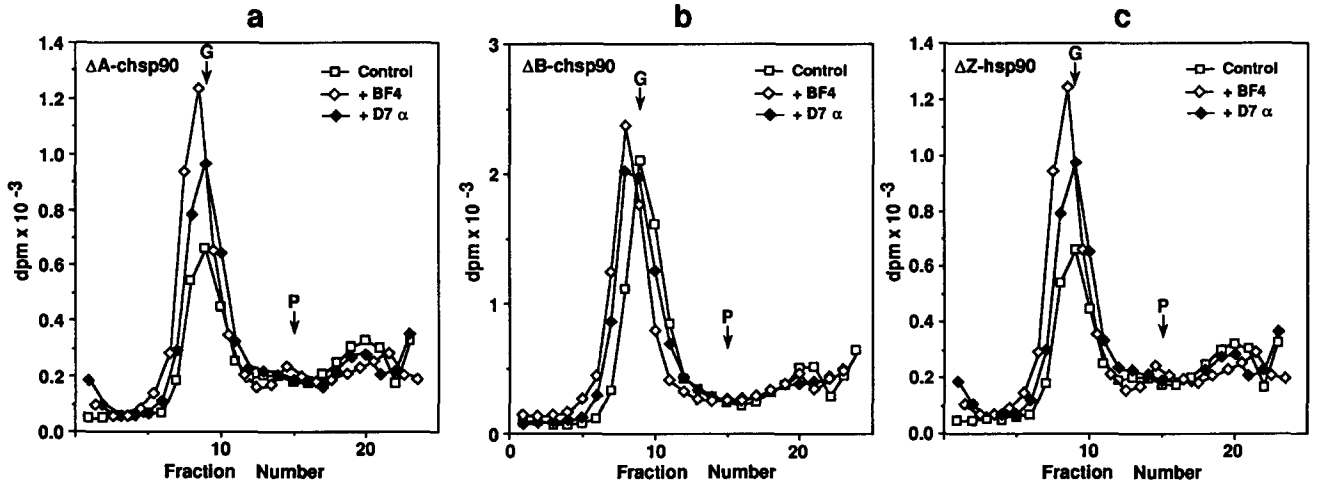

Fig. 4. Sedimentation analysis of [^{3}H]TA-bound hGR coexpressed with mutated chsp90s. Effect of D7α and BF4 antibodies. Cytosol aliquots (37 μg for A- and Z-deleted chsp90s or 63 μg for B-deleted chsp90) were diluted to 100–200 μl with GTED buffer containing protease inhibitors and incubated for 2 h without additives (□) or with 5/10 μl of D7α (◆) or 10/20 μl of BF4 (◇) antibodies (ascites fluids). They were layered on sucrose/glycerol gradients containing 10% v/v glycerol and analyzed as described in Experimental (a) A-deleted chsp90,(b) B-deleted chsp90 and (c) Z-deleted chsp90.

Mutants B and Z, but not mutant A, interact with the hGR

A rabbit polyclonal anti-hGR antibody was utilized to prepare a protein-A-sepharose resin able to adsorb hGR-containing complexes. The propensity of mutated chsp90s to stick to IgG was attenuated by two preliminary successive adsorptions to IgG control resin before the definitive parallel analysis on specific and control resin. Figure 5 shows the hsp90s immunoadsorbed in [^{35}S]Met cytosols [Fig. 5(a)] and in unlabeled cytosols from cells infected for 48 h [Fig. 5(b)].

In [^{35}S]Met labeled cytosols, chsp90 was preferentially labeled with respect to other cellular components, consistent with its very high synthesis rate beyond 24 h after infection. At this time, [^{35}S]GR was expected to interact predominantly with chsp90. Data from pulse–chase experiments in S49 cells [24] are consistent with preferential association of newly synthesized chsp90 and GR and with no exchange of hsp90 in the complexes. (In unlabeled cytosols from 24 h-cultured cells, the 40% value corresponded to a mean index of GR complexed with chsp90 and synthesized from zero time to 24 h.) The immunoabsorbed [^{35}S]GR and [^{35}S]chsp90 retained were directly detected by gel autoradiography [Fig. 5(a)].

When using unlabeled cytosols from 48 h-cultured cells, total GR complexes were analyzed and immunoabsorbed chsp90s were detected by Western blot using the AC19 antibody [Fig. 5(b)].

In [^{35}S]Met cytosols, wild-type and Z- and B-deleted chsp90s were significantly retained on the hGR matrix (less efficiently for the B-deleted mutant). No significant adsorption was recorded for the A-deleted mutant

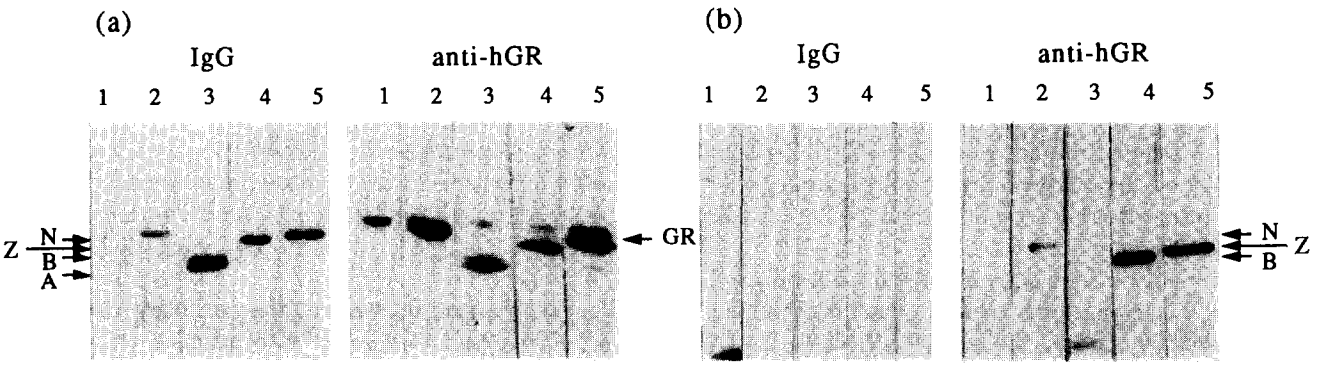

Fig. 5. Immunoadsorption of hsp90s on an anti-GR matrix. (a) 0.6 ml (0.9 to 2.4 mg protein) of various [^{35}S]Met Sf9 cell cytosols were incubated twice with 0.2 ml of control IgG matrix. Half of the resulting supernatant was incubated with 0.1 ml of IgG control resin while the other half was incubated with anti-hGR resin. Eluates obtained from washed resins were analyzed by SDS-PAGE as described in Experimental and chsp90s were detected by autoradiography. (b) 0.7 to 1.5 mg protein of unlabeled cytosol aliquots were treated as described in a. The chsp90s were detected by Western blot analysis, using AC19 antibody. 1: hGR expressed alone; 2: hGR expressed with wild-type chsp90; 3: hGR expressed with A-deleted chsp90; 4: hGR expressed with B-deleted chsp90; 5: hGR expressed with Z-deleted chsp90.

[Fig. 5(a)]. We quantified the band intensities obtained on the gel autoradiogram and determined hsp90/hGR ratios (after substraction of IgG-adsorbed hsp90). Values close to 2 were obtained for B- and Z-deleted chsp90s, in agreement with the reported stoichiometry of two molecules of hsp90 per hGR molecule in the hetero-oligomeric complex [25, 26]. A smaller value of 1.54 was obtained for wild-type chsp90. Two explanations can be proposed for this smaller value. The wild-type complex is less stable than complexes formed with mutated chsp90s and some dissociation occurs during washing. Alternatively or concomitantly, a small amount of GR interacts with unlabeled insect hsp90. This implies that B- and Z-deleted chsp90 may form some complexes containing more than two chsp90 molecules per GR. This assumption is compatible with detection of some mutated chsp90 in the high molecular weight fractions of glycerol/sucrose gradients in addition to the chsp90 dimer (not shown).

Results obtained with unlabeled cytosols were coincident. We observed strong bands for the Z- and B-deleted chsp90s [Fig. 5(b)], consistent with the formation of Z- and B-deleted chsp90 complexes with chsp90/GR ratios > 2. For the wild-type chsp90, only a faint band was detected. For insect hsp90, no band was detected, consistent with the lower reactivity of AC19 for insect hsp90 than for chsp90.

DISCUSSION

The data presented here suggest that the A region is critically involved in the interaction of hsp90 with the hGR. The observation that two other mutants are still able to interact with the hGR without conferring steroid binding ability, underlines the importance of other regions of the hsp90 in establishing a proper conformation for the GR LBD, either directly or by maintaining the appropriate hsp90 structure.

Structure and role of the A region in interactions with the GR

Immunoadsorption results show that the A-deleted chsp90 does not interact with the hGR, thus demonstrating the importance of this region of hsp90 in the formation of hetero-oligomeric complexes. In this regard, the A-deleted mutant resembles the *E.Coli* hsp90 analog, where this charged region is essentially absent (corresponding to amino-acids 236 to 285 in chsp90α) [14]. The expression of the LBD of the GR in this microorganism did not generate a steroid binding GR molecule [27], consistent with our data. Nevertheless, the immunoadsorption technique we used involves many washing steps which could cause dissociation of unstable hGR–hsp90 complexes and the use of another technology under milder conditions or *in vivo* analysis would be useful to confirm the necessary role of the A region in hsp90–receptor interaction. Moreover, it cannot be excluded that deletion of part of the hsp90 may modify the conformation of the remaining molecule and interfere with functions accomplished by the other hsp90 subregions.

The mechanism by which the A region may act is still unknown although its high content of negatively charged amino-acid residues would allow ionic interactions with positively charged hGR segments of the LBD. Although GR nuclear localization region NL1 is rich in basic amino-acids and is not accessible to antibody raised against this region, when GR is in the 8S hetero-oligomeric form [28, 29], NL1 did not participate in hsp90–GR interaction which is in contrast to data obtained with the estrogen receptor [12]. We do not yet know the tridimensional structure of the hGR LBD and the arrangement of various positively charged regions. Since two A regions are probably present on the dimer surface, the fact that only one molecule of receptor interacts with the hsp90 dimer raises the question of what is the relative position of the two A regions. Perhaps, they are in close proximity or even involved in the multipoint anchoring of the GR LBD [11].

The A region is also crucial for the recognition by the BF4 antibody and contains at least part of the BF4 epitope as shown by the lack of displacement by BF4 of the free A-deleted mutant (N.B., unpublished observations and this work) and by the BF4 reactivity towards a chimeric hsp90β protein containing the A region of hsp90α instead of the unreactive A region of hsp90β (30 and X. Meng and M. G. Catelli, personal communication). The reported stoichiometry of BF4 binding, one BF4 per hsp90 dimer [25], suggests that the geometry of the A regions allows only one molecule of voluminous proteins as antibody and/or receptor to interact.

Influence of B and Z deletions on hGR steroid binding properties

The lack of 8S heterooligomeric hGR displacement by chsp90 specific antibodies on sucrose gradients and the detection of B- and Z-deleted chsp90s on anti-hGR immunoadsorbant, demonstrate that complexes with the hGR can be formed without maintaining the proper folding of the hGR LBD for hormone binding. As the transformation step triggered by the hormone *in vivo* dissociates hsp90 and relieves hsp90 inhibition of the receptor function, the hetero-oligomeric complexes formed with B- and Z-deleted chsp90s, unable to bind hormone, may not be dissociated and activated, as already described for deletions in the hGR LBD [11], or with an antagonist ligand (RU486) [4].

This study describes a tool for analyzing the functions of hsp90 subregions with respect to various associated proteins: steroid receptors, v-erb A oncogene protein [31], the dioxin receptor [32], p59/HBI [33] and others.

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