Distinct functions of the 90 kDa heat-shock protein (hsp90) in oestrogen and mineralocorticosteroid receptor activity: effects of hsp90 deletion mutants

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Recent studies have confirmed that the 90 kDa heat-shock protein (hsp90) interacts both *in vitro* and *in vivo* with steroid receptors, encouraging further detailed physicochemical and functional analysis of its chaperone role. Thus, to explore the relationship between hsp90 and receptors, the baculovirus system was used to overexpress the chick hsp90 α (chsp90) along with the chick oestradiol receptor (cER) or the human mineralocorticosteroid receptor (hMR). These receptors were able to form 9 S complexes with chsp90, demonstrating the association of the co-expressed recombinant proteins. Three mutants of chsp90 (Δ A, Δ B and Δ Z) have been created by deletion of the A (residues 221–290) and B (530–581) regions, rich in charged amino acids, and the Z (392–419) region, a putative leucine zipper. After co-expression, anti-receptor antibodies immunoprecipitated the cER

or hMR complexed with the wild-type chsp90, the Δ B or the Δ Z mutant, but not with the Δ A chsp90, indicating that deletion of the A region of chsp90 leads to a lack of interaction with these receptors. The hormone binding capacity of the cER was unaffected after its co-expression with each of the three mutants. In contrast, the hMR co-expressed with the Δ B mutant failed to bind aldosterone, a finding confirmed *in vivo* by the absence of hormone-induced hMR nuclear translocation. Thus the B region is required for high-affinity ligand binding by the hMR. Our results suggest that the A region (but not the B or Z regions) is involved in binding of chsp90 to the cER and hMR, while the B region is essential for hormone binding by the hMR, consistent with a chaperone function for hsp90.

INTRODUCTION

The 90 kDa heat-shock protein 90 (hsp90), one of the most abundant and ubiquitous cellular proteins, belongs to the family of stress-inducible proteins [1]. Hsp90 is known to interact with several proteins including various kinases [2–4], components of the cytoskeleton [5,6], elements of the protein synthesis machinery [7] and intracellular receptors [8–12], suggesting some general function of hsp90 in cellular physiology. A chaperone role for hsp90 has been proposed [13]. The demonstration that hsp90 enhances the renaturation of some proteins *in vitro* provides additional support for its role in protein folding [14].

The formation of complexes between hsp90 and intracellular receptors such as those for oestradiol [15], progesterone [10], androgen [16], glucocorticosteroids [17], mineralocorticosteroids [12] and dioxin [11] has been demonstrated in the cytosol of target tissues. In accordance with the numerous *in vitro* studies, direct evidence for an *in vivo* interaction of hsp90 with steroid receptors has recently been established [18], encouraging further detailed physicochemical and functional studies of hsp90–receptor complexes. In their non-transformed state, steroid receptors are recovered as hetero-oligomers including a dimer of hsp90 [19,20] and other receptor-associated proteins such as hsp70 [21] and the immunophilin p59 [22,23], whose role in receptor function is not yet understood. Positive and negative modulation by hsp90 of steroid receptor activity has been described. Hsp90 can maintain the receptor in a non-functional state; upon hormone binding and hsp90 release, the receptor

acquires the ability to bind specific DNA sequences [24]. With respect to gluco- and mineralo-corticosteroid receptors, biochemical data suggest that hsp90 is essential for their high-affinity ligand binding [25–29]. Finally, hsp90 also plays a positive role in the hormone signal transduction pathway, since it confers on the receptor the ability to become an active hormone-induced transcription factor [30]. Various biochemical studies of steroid receptors have shown that their ligand binding domain is directly involved in the interaction with hsp90 [31–33].

To better understand hsp90 function, we have investigated the *in vivo* association of the chick hsp90 α (chsp90) with two members of distinct subclasses belonging to the steroid receptor gene superfamily: the chick oestradiol receptor (cER) and the human mineralocorticosteroid receptor (hMR). We used the baculovirus system for overexpressing chsp90 and steroid receptors efficiently and for investigating their *in vivo* assembly by co-expression of the recombinant proteins in the same insect cell. To determine the regions of hsp90 implicated in its interaction with the receptor, three deletion mutants were constructed. In the first the A region, which is rich in negatively charged residues and has been proposed as a potential candidate for receptor interaction [34], was deleted. Two further constructs showed deletion of the B and Z regions respectively, which are heptad leucine repeats representing putative domains of protein–protein contact.

In this study, we provide evidence that the co-expressed chsp90 can associate with the cER or hMR. Two regions of hsp90 that are important for steroid receptor function have been identified; one is involved in the binding of the protein to the receptors, and

Abbreviations used: (c)hsp90, (chick) 90 kDa heat-shock protein; cER, chick oestradiol receptor; hMR, human mineralocorticosteroid receptor; AcNPV, *Autographa californica* nuclear polyhedrosis virus; DCC, dextran-coated charcoal; GR, glucocorticosteroid receptor.

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the other is essential for the correct conformational structure of the ligand binding domain of the hMR.

MATERIALS AND METHODS

Cell culture

Insect cells of *Trichoplasia ni* (SB1-4), designated High Five cells (British Bio-technology Products Ltd., Oxford, U.K.), or Sf9 cells were grown at 27 °C and maintained in TNM-FH Insect Medium (Sigma) containing 10% (v/v) stripped foetal calf serum. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant baculoviruses were used to infect cells at a multiplicity of infection of > 10 as previously described [35].

Construction of recombinant transfer vectors and recombinant baculoviruses

The chick hsp90 sequence [34] was digested with *Bss*HII and *Mae*I restriction enzymes and the fragment containing the chsp90 coding sequence plus 26 bp of 5' and 266 bp of 3' untranslated sequences was then inserted into the *Bam*HI site of the baculovirus transfer vector pVL941 after filling in all protruding ends. Deletions of the A region (amino acids 221–290) and the B region (amino acids 530–581) were obtained by oligonucleotide-directed mutagenesis of the *Pst*I–*Eco*RI fragment in M13mp9 or of the *Eco*RI–*Pst*I fragment in M13mp8 respectively, before their reintroduction into the *Bss*HII–*Mae*I fragment of the chsp90 cDNA. Recombinant transfer vectors were selected by hybridization with the mutated oligonucleotide. The deletion of the Z region (amino acids 392–419) was performed using an oligonucleotide-directed *in vitro* mutagenesis system as described by the supplier (Amersham). All constructs were sequenced to confirm the deduced primary structures of the mutants. cER cDNA [36] was digested with *Eco*RI; the fragment containing the entire cER coding sequence plus 9 bp of 5' and 120 bp of 3' untranslated sequences was then inserted into the *Bam*HI site of the transfer vector. hMR DNA [37] was inserted into pVL941 as described previously [35].

The recombinant baculoviruses AcNPV-chsp90, AcNPV-chsp90ΔA, AcNPV-chsp90ΔB, AcNPV-chsp90ΔZ, AcNPV-cER and AcNPV-hMR, which contain the chsp90, the deleted A region mutant of chsp90 (ΔA), the deleted B region mutant of chsp90 (ΔB), the deleted Z region mutant of chsp90 (ΔZ), the cER and the hMR sequences respectively, under the transcriptional control of the polyhedrin promoter, were produced by *in vivo* recombination.

Expression and partial purification of baculovirus recombinant proteins

The expression of recombinant proteins was performed in Sf9 cells essentially as in [35]. Overexpressed ER was partially purified on preparative high-salt density gradients. The fractions containing [³H]oestradiol binding activity were pooled and dialysed against TEG buffer [20 mM Tris/HCl, 1 mM EDTA, 10% (v/v) glycerol, pH 7.4, at 20 °C]. The overproduced chsp90 was highly purified on a specific BF4 immunoabsorbent column as in [20]. Both purified proteins were used to perform *in vitro* reassociation studies.

Metabolic labelling and immunoprecipitation of cER and hMR complexes

High Five cells (18 × 10⁶ cells per plate) were infected with AcNPV-cER virus or AcNPV-hMR virus in combination with

AcNPV wild-type, chsp90 or chsp90 mutant viruses. Mock-infected cells were used as controls. After 24 h of infection, the cells were incubated for 1 h with methionine-free TNM-FH medium and then with 300 µCi of [³⁵S]methionine per plate for 6 h. Cells were rinsed with PBS and then homogenized with a glass/glass Potter-type homogenizer in TEGW buffer (TEG buffer containing 20 mM sodium tungstate) supplemented with protease inhibitors (1 mM PMSF, 5 µg/ml antipain, 5 µg/ml leupeptin). Homogenates were centrifuged at 12000 g for 15 min at 4 °C and the supernatants were considered as cytosol fractions.

Cytosols were subsequently used for immunoprecipitation assays. Cytosol aliquots (100 µl) were incubated overnight with 2 µl of monoclonal anti-ER antibody JS 34/32 [38] or 5 µl of the purified monoclonal anti-hMR antibody FD4 (1 mg/ml) [39], or with NS1 (Sigma) or MOPC31C (1 mg/ml) as rat and murine IgG controls respectively. Immune complexes were subsequently adsorbed on Protein G–Sepharose (20 µl) (Pharmacia) in an end-over-end rotator for 3 h at 4 °C. The pellets were washed three times in TEGW buffer containing 50 mM NaCl/0.1% Tween 20, directly boiled for 4 min and the samples were analysed by SDS/PAGE (7.5% gels). The gels were fixed with propan-2-ol/water/acetic acid (25:65:10, by vol.) for 30 min and then treated with Amplify (Amersham Corp.), dried and subjected to autoradiography at –80 °C. The relative amounts of [³⁵S]-methionine-labelled proteins were calculated by scanning the radioactivity of the dried gels with an automatic analyser (Multitrac Master Model LB285; Berthold Analytical Instruments, Nashua, NH, U.S.A.) or by quantifying the absorbance of the autoradiographic signals using a Millipore Bioimage Electrophoresis analyser.

Binding assays

Cytosol fractions were incubated for 4 h at 4 °C with tritiated steroid in the absence or presence of a 100-fold excess of unlabelled steroid. Samples were treated with dextran-coated charcoal (DCC) as previously described [12]. [³H]Tamoxifen aziridine was used to label the cER covalently and, after DCC treatment, aliquots were analysed by SDS/PAGE.

SDS/PAGE and Immunoblotting

Samples were subjected to electrophoresis on a 7.5% polyacrylamide gel in the presence of SDS. Gels were transferred to nitrocellulose membranes that were subsequently blocked with 10% non-fat dried milk in PBS for 1 h at room temperature. The membranes were incubated overnight at 4 °C with the appropriate antibody. The detection of the protein was realized using the Vectastain ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA, U.S.A.).

Gel retardation assays

A 37 bp synthetic oligonucleotide containing the palindromic sequence of the *Xenopus* ERE vitellogenin A2 gene was purified, ³²P-labelled and incubated for 15 min at 25 °C with cellular extracts containing the cER. Protein–DNA complexes were separated from free DNA by non-denaturing electrophoresis in a 4.5% acrylamide/bisacrylamide (39:1, w/w) gel. All procedures were essentially the same as described in [40].

Sedimentation analysis on density gradients

Aliquots of labelled cytosol were layered on a 5–20% sucrose gradient prepared in TEGW buffer and centrifuged at 385000 g for 105 min in a Beckman VTi65.2 rotor, or at

217000 *g* for 17 h in a Beckman SW60 rotor. Sedimentation coefficients were determined using glucose oxidase of fungal origin (Boehringer Mannheim; $s_{20,w} = 7.9$ S) and horseradish peroxidase (Sigma type VI; $s_{20,w} = 3.6$ S) as internal standards.

ELISA

Microtitre plates were coated with 100 μ l aliquots of ultracentrifugation fractions for 1 h at 37 °C. After washing with PBS containing 0.1 % Tween 20 (PBST), purified anti-(chick hsp90) BF4 antibody [41] was added to the wells and incubated for 1 h at 37 °C. After washing with PBST, alkaline phosphatase-conjugated rabbit anti-rat IgG (Sigma) was added for 1 h at 37 °C. chsp90 was detected by adding a substrate solution (1 mM *p*-nitrophenyl phosphate in 1 M Tris/HCl, pH 8.0). Absorbances were read at 405 nm on a microplate EL308 reader (Bio-Tek Instruments, OSI, Elancourt, France).

Immunohistochemical studies

High Five cells were infected with recombinant baculoviruses. After 24 h of infection, the cells were incubated overnight with or without 100 nM aldosterone, rinsed with PBS and fixed with Bouin's fluid. After paraplast embedding, tangential sections were processed for immunohistochemistry as in [39]. FD4 or BF4 were used as primary antibodies and the avidin/biotin/peroxidase or alkaline phosphatase system (Vector Laboratories, Burlingame, CA, U.S.A.) was used to immunodetect the hMR and hsp90 proteins respectively. After immunostaining, histological examination was performed by two independent observers in 'blind' experiments, each counting about 1000 cells as in [39]. The positive cells were classified into three different labelling categories according to the localization of the immunoreactive proteins (exclusively cytoplasmic, cytoplasmic plus nuclear or exclusively nuclear staining). The results were tested for statistical significance using the χ^2 test.

RESULTS

Characterization of chsp90 and its three mutants

We have studied chsp90 expression in insect cells after infection with the baculovirus AcNPV-chsp90, and optimized conditions for the production of the recombinant protein. A 90 kDa protein

was readily detected on a Coomassie Blue-stained gel 24 h post-infection. To estimate the amount of avian hsp90 produced, cellular extracts of infected cells were subjected to electrophoresis and compared with known quantities of highly purified chick oviduct hsp90. The quantity of chsp90 expressed in 25×10^3 cells is approximately equal to 1 μ g of pure chsp90, corresponding to about 40 % of the total soluble protein of the insect cells. The recombinant chsp90 was analysed by ultracentrifugation and detected by ELISA with BF4, a monoclonal anti-hsp90 antibody which recognizes only the chick protein [41]. The recombinant chsp90 sedimented at 6.4 S; its Stokes radius determined by gel filtration on Ultrogel ACA34 was 6.4 nm (results not shown). Altogether the recombinant chsp90 has a calculated molecular mass of 173 kDa, consistent with a dimeric form as already reported for the native protein [20].

Three deletion mutants of chsp90 have been constructed. In the ΔA mutant the negatively charged A region (amino acids 221–290) was deleted; of the 70 amino acids deleted, 53 (76 %) are charged residues [34]. The ΔB mutant was constructed by deleting the highly charged B region (56 % charged residues) between residues 530 and 581; most of the deleted residues are hydrophobic. This B region is similar (78 % sequence identity) to that of the murine hsp84 extending from amino acids 558 to 588, which corresponds to a large amphipathic helix with a basic helix-loop-helix structure. This region may present a leucine zipper structure [42]. The ΔZ mutant corresponds to the deletion of the potential leucine heptad repeat in the middle of the molecule between amino acids 392 and 419. The amphipathic helix predicted in the Z region contains repeated hydrophobic residues, consistent with a leucine zipper structure [43], which fits with a possible dimerization domain.

Scheme 1 summarizes the physicochemical characteristics of the three chsp90 mutant proteins. They were overexpressed at the same level as the wild-type chsp90, as determined by Coomassie Blue staining of polyacrylamide gels (results not shown). The ΔB and ΔZ mutants were eluted in the void volume of the AcA34 column, suggesting aggregation and/or polymerization processes which are enhanced after longer infection times. In contrast with the ΔA mutant, they were isolated from the cytosol not only as dimers, supporting the hypothesis that the B and Z regions are possible dimerization domains. Various immunological techniques showed that the anti-chsp90 antibody BF4 did not bind

Protein	Deleted amino acids		Apparent molecular mass (kDa)	$s_{20,w}$ (S)	R_s (nm)	Antibody recognition	
						BF4	AC88
chsp90	–		90	6.5	6.4	+	+
ΔA	221–290		83	6.6	ND	0	+
ΔB	530–581		85	5.5	*	+	+
ΔZ	392–419		87	6.4	*	+	+

Scheme 1 Schematic representation of chsp90 and its mutants

The construction of the wild-type chsp90 and its mutants is described in the Materials and methods section. The apparent molecular mass corresponds to that established by SDS/PAGE. Sedimentation coefficients ($s_{20,w}$) were measured in the present study. The Stokes radius (R_s) was calculated from gel filtration on an AcA34 Ultrogel column. * indicates a propensity of the mutants to aggregate, since they were eluted with the void volume of the column. The epitope mapping of the BF4 antibody has been established using Western blot analysis, ELISA and immunohistochemistry. AC88 was used as a control. ND, not determined.

Figure 1 Characterization of the recombinant cER from baculovirus-infected cells

(a) Affinity labelling of the recombinant cER. Cytosol aliquots of infected Sf9 cells were labelled with 100 nM [3 H]tamoxifen aziridine in the absence (lane 1) or presence (lane 2) of a 100-fold excess of unlabelled tamoxifen for 4 h at 4 °C. Free steroid was removed by DCC treatment. SDS/PAGE analysis was performed as described in the Materials and methods section. (b) Western blot analysis. Samples of 100 μ g of protein from infected (lane 1) or uninfected (lane 2) cells were subjected to SDS/PAGE and immunoblotted for detection of the cER with H226 antibody (1:100 dilution). (c) Specific binding of the cER to oestrogen response elements. [32 P]ERE oligonucleotide (lane 1) (0.5 ng) was incubated with 20 μ g of cytosol protein from infected cells containing cER (60 fmol) (lanes 2–4) or from non-infected cells (lane 5). H226 antibody (1 μ g) (lane 3) or a 100-fold excess of unlabelled ERE (lane 4) was added to the binding assay. Lane M contains molecular mass markers.

the ΔA mutant, indicating that the epitope of BF4 is located in the A region (amino acids 221–290). Taking into account the fact that this antibody recognition is restricted to the chick protein, and considering the sequences of hsp90s from different species, it is likely that the epitope of BF4 corresponds to the limited and non-conserved sequence EEKEEKTED (amino acids 242–250 in chsp90 α).

Characterization of recombinant steroid receptors

Infection of Sf9 cells with the recombinant baculovirus AcNPV-cER resulted in the expression of the cER as early as 24 h post-infection. Scatchard plots indicated a single class of specific binding sites for [3 H]oestradiol with an apparent dissociation constant $K_d = 1.8$ nM, which is comparable with that reported for the endogenous chick oviduct ER [44]. The binding capacity in the cytosol fraction of infected cells after 48 h of infection was 32.8 ± 6.5 pmol/mg of protein (mean $\pm$ S.E.M.; $n = 10$), representing about 10^6 sites/cell. The molecular mass of the expressed receptor was determined by SDS/PAGE after labelling the cytosol with [3 H]tamoxifen aziridine in the absence (Figure 1a, lane 1) or presence (Figure 1a, lane 2) of a 100-fold excess of unlabelled ligand. The autoradiogram revealed a single band at 66 kDa, which agrees with the strong signal observed in a Western blot using the monoclonal anti-ER antibody H226 in infected cells (Figure 1b, lane 1) but not in uninfected cells (Figure 1b, lane 2), confirming the identity of the cER produced. In a gel shift mobility assay (Figure 1c), the cER specifically retarded the migration of an oligonucleotide containing the sequence of the *Xenopus* ERE vitellogenin A2 gene (lane 2). Addition of H226 antibody decreased the mobility of cER–ERE complexes (lane 3). No retarded band was observed with cellular extracts of mock-infected cells (lane 5). These cER characteristics are comparable with those obtained for the human ER [45,46].

Figure 2 Recombinant hMR or cER is associated with co-expressed chsp90

Cytosols from cells infected with AcNPV-chsp90 and either AcNPV-hMR or AcNPV-cER were prepared in TEGW buffer labelled with [3 H]aldosterone (a) or [3 H]oestradiol (b) respectively. Samples (11 μ g and 2 μ g of protein for a and b respectively) were incubated with a rat control ascite (NS1) (■, ●) or the monoclonal antibody BF4 (□, ○) for 4 h at 4 °C and layered on a 5–20% sucrose/glycerol gradient. Ultracentrifugation was performed in a VT65.2 rotor for 1 h 45 min at 385 000 g. Internal standards were: GO, glucose oxidase ($s_{20,w} = 7.9$ S); PO, horseradish peroxidase ($s_{20,w} = 3.6$ S).

The recombinant hMR was also expressed as a functional protein in the baculovirus-infected cells as previously described [35,40], but the amount of expressed receptor as assessed by binding assays (approx. 2 pmol/mg of protein) was at least 10 times lower than that of the cER.

Recombinant chsp90 is associated with recombinant steroid receptors

The baculovirus-expressed steroid receptors (hMR or cER) sedimented at 8–9 S, consistent with their association with the endogenous insect hsp90. Indeed, after addition of a polyclonal anti-peptide antibody (B174) directed against the C-terminal region of the hsp90, steroid–receptor complexes were displaced into the 10–11 S region of the gradient ([35]; results not shown). When the hMR or cER was co-expressed with the chsp90 (Figure 2), they also formed 9 S entities that were shifted by addition of the anti-chsp90 BF4 antibody. These results clearly demonstrate that the recombinant chsp90 forms an hetero-oligomeric complex with steroid receptors. Thus the baculovirus expression system was appropriate for studying their protein–protein interactions in detail.

Deletion of the A region prevents interaction of the chsp90 with the cER and hMR

In an attempt to study the reassociation of receptor and chsp90, we performed preliminary experiments by mixing partially purified cER and chsp90. However, such *in vitro* reassociation has been so far unsuccessful. Therefore, to address the question of the *in vivo* assembly of the steroid receptor and to identify the region of the chsp90 implicated in its interaction with the receptor, the cER or the hMR was co-expressed with either chsp90 or one of the three mutants ΔA , ΔB and ΔZ . After co-infection with recombinant baculoviruses and [35 S]methionine metabolic label-

Figure 3 Co-expression of hMR and chsp90 mutants

High Five cells infected by AcNPV-hMR together with AcNPV-chsp90 (lane 1), AcNPV-chsp90 Δ A (lane 2), AcNPV-chsp90 Δ B (lane 3) or AcNPV-chsp90 Δ Z (lane 4) were metabolically labelled with [35 S]methionine. Cytosolic extracts were analysed on 7.5% acrylamide gels. (a) Autoradiography of labelled proteins (80 μ g); (b) and (c) Western blots of the same extracts: 80 μ g of protein probed with FD4 antibody (b) or 20 μ g of protein revealed by BF4 antibody (c). The positions of markers (kDa) (Rainbow standards) are indicated on the left of the figure.

Figure 4 Immunoprecipitation of cER complexes

[35 S]Methionine-labelled cytosolic proteins from cells infected by recombinant baculoviruses were incubated with the monoclonal anti-ER antibody JS34/32 and subsequently with Protein G-Sepharose as described in the Materials and methods section. Immunoprecipitated samples were analysed by SDS/7.5%-PAGE and by autoradiography. (a) Cells infected by AcNPV-cER alone (cER) or AcNPV-chsp90 alone (chsp90). (b) Cells infected by AcNPV-cER together with AcNPV (cER), AcNPV-chsp90 (H), AcNPV-chsp90 Δ A (Δ A), AcNPV-chsp90 Δ B (Δ B) or AcNPV-chsp90 Δ Z (Δ Z).

ling, cellular extracts were prepared in the absence of hormone in low-salt buffer containing sodium tungstate to preserve the integrity of the hsp90-receptor complexes. Such a co-expression of hMR and chsp90 mutants is illustrated in Figure 3. The signal intensity of the hMR seen at 120 kDa appeared similar under each co-infection condition (Figure 3a). Moreover, the overall expression of the wild-type and mutant chsp90s, recovered at their expected size, was greater than that of the receptor. Cellular extracts were analysed by Western blot with the anti-hMR antibody FD4 (Figure 3b) or the anti-chsp90 antibody BF4 (Figure 3c). All chsp90 mutants were reactive with BF4 except

Figure 5 Immunoprecipitation of hMR complexes

[35 S]Methionine-labelled cytosolic proteins from cells infected by AcNPV-hMR together with AcNPV (C; lanes 1 and 2), AcNPV-chsp90 (H; lanes 3 and 4), AcNPV-chsp90 Δ A (Δ A; lanes 5 and 6), AcNPV-chsp90 Δ B (Δ B; lanes 7 and 8) or AcNPV-chsp90 Δ Z (Δ Z; lanes 9 and 10) were incubated with either FD4 (lanes 1, 3, 5, 7 and 9) or MOPC31C (lanes 2, 4, 6, 8 and 10) antibody and subsequently processed as previously described. Immunoprecipitated samples were analysed by SDS/7.5%-PAGE and by autoradiography.

the Δ A mutant (Figure 3c, lane 2), consistent with the localization of the BF4 epitope in the A region of chsp90.

Immunoprecipitation assays were performed to study the interactions between the receptors and the chsp90 mutants. Immune-isolated complexes were analysed by electrophoresis. Figure 4(a) demonstrates that the anti-ER JS34/32 antibody immunoprecipitates the receptor (cER) but not the hsp90 (chsp90), demonstrating the specificity of this antibody. Figure 4(b) shows the immunoprecipitated chsp90-cER complexes. When cER was expressed with polyhedrin used as a control, JS34/32 precipitated only one band corresponding to the receptor. This antibody also immunoprecipitated the cER associated with wild-type chsp90, the Δ B or the Δ Z mutant. In contrast, the Δ A mutant was not specifically co-precipitated with the cER, indicating that the A region of chsp90 is involved in the interaction with this steroid receptor.

A similar immunoprecipitation assay was carried out to examine the association between the hMR and chsp90 using the anti-hMR FD4 antibody [39]. Figure 5 shows that FD4 specifically immunoprecipitated the hMR (odd-numbered lanes) and also co-precipitated wild-type chsp90 (lane 3) and the Δ B (lane 7) and Δ Z (lane 9) mutants. In contrast, the signals corresponding to the Δ A chsp90 immunoprecipitated with FD4 or with MOPC31C control antibody (lanes 5 and 6) were not different, suggesting that the Δ A mutant was not physically bound to the receptor. To validate this assumption, six independent experiments were quantified by scanning the radioactive signals of the gels or analysing the absorbance of the autoradiographic spots; both analyses gave identical results. As shown in Table 1, the amount of chsp90 protein specifically immunoprecipitated by the anti-hMR FD4 antibody (relative to the co-precipitated hMR and taking into account the non-specific trapping) was significantly lower for the Δ A mutant ($P < 0.02$) than for the wild-type chsp90, the Δ B and the Δ Z mutants. This result clearly demonstrates that the A region of chsp90 is also involved in the interaction with the hMR.

Table 1 Quantitative analysis of specific hsp90 proteins immunoprecipitated by FD4

Immunoprecipitation experiments similar to that presented in Figure 5 were performed and the signals corresponding to the hMR and the chsp90 proteins were quantified by measuring directly their radioactivity and the absorbance of the autoradiographic spots. The amount of hsp90 specifically immunoprecipitated by the monoclonal anti-hMR antibody was calculated after subtraction of the non-specific retention and compared with the quantity of co-precipitated hMR. Values are means $\pm$ S.E.M. from six independent experiments. Statistical analysis has been performed using Student's *t* test; **P* < 0.02 compared with wild-type.

Protein	Specific hsp90 immunoprecipitation
chsp90	0.59 $\pm$ 0.16
Δ A mutant	0.14 $\pm$ 0.03*
Δ B mutant	0.69 $\pm$ 0.13
Δ Z mutant	0.71 $\pm$ 0.10

Table 2 Effect of mutated hsp90 on the steroid binding capacity of hMR and cER

Aldosterone or oestradiol binding assays were carried out using the cytosol fractions of cells infected with AcNPV-hMR or AcNPV-cER together with AcNPV-chsp90 (hsp90), AcNPV-chsp90 Δ A (Δ A mutant), AcNPV-chsp90 Δ B (Δ B mutant), AcNPV-chsp90 Δ Z (Δ Z mutant) or AcNPV (used as a control). Results are expressed as percentages of steroid binding measured in controls (means $\pm$ S.E.M. of five independent experiments); 100% corresponds to 10.70 $\pm$ 0.34 pmol/mg of protein (ER) or 1.17 $\pm$ 0.24 pmol/mg of protein (MR).

Protein	Hormone binding capacity (%)	
	Aldosterone	Oestradiol
chsp90	100.4 $\pm$ 12.4	95.9 $\pm$ 3.6
Δ A mutant	103.8 $\pm$ 21.0	110.3 $\pm$ 20.4
Δ B mutant	11.6 $\pm$ 3.3	114.3 $\pm$ 16.7
Δ Z mutant	102.4 $\pm$ 5.0	93.5 $\pm$ 14.0

Influence of the chsp90 mutants on steroid receptor function

We measured the ability of the hMR and cER to bind their cognate ligands after their co-expression with wild-type chsp90 or each of the three mutant chsp90s. Table 2 shows that, when co-expressed with recombinant chsp90, the hMR and cER bound aldosterone and oestradiol respectively to the same extent as the controls. Similarly, the binding capacity of both receptors co-expressed with the Δ A mutant was not different from the control values. This is consistent with the results of immunoprecipitation assays, which demonstrated that both receptors were not associated with this mutant but rather bound to the endogenous insect hsp90. There was no significant difference in the hormone binding capacity of both the hMR and cER co-expressed with the Δ Z mutant. Surprisingly, when the hMR was expressed with the Δ B mutant, only 10% of the hormone binding capacity was recovered, indicating that the hMR complexed to the Δ B chsp90 has lost its ability to bind aldosterone. This was not the case for the cER; the oestradiol binding capacity of the receptor was the same with all hsp90 mutants.

The Δ B mutant precludes the aldosterone-induced nuclear translocation of the hMR

The presence of the recombinant hMR and the hsp90s in the baculovirus co-infected cells was examined by means of immunohistochemical methods using FD4 and BF4 antibodies respectively (Figure 6). A double-labelling technique (peroxidase for hMR detection and alkaline phosphatase for hsp90 detection) was used to show that both proteins are co-expressed *in vivo* in the same cells. However, it is noticeable that approx. 20% of the cells expressed only one of the recombinant proteins. The recombinant chsp90 (Figure 6a) and its mutants (e.g. Δ B in Figure 6b) were localized in the cytoplasmic compartment. In the absence of hormone, the hMR was also almost exclusively localized in the cytoplasmic compartment (Figures 6c and 6d).

Figure 6 Immunodetection of hMR and chsp90 proteins in co-infected High Five cells

High Five cells were infected by AcNPV-hMR together with either AcNPV-chsp90 (a, c, e) or AcNPV- Δ B (b, d, f). At 24 h post-infection, the cells were incubated overnight without (a–d) or with (e, f) 100 nM aldosterone. The cells were processed for immunohistochemistry as described in the Materials and methods section using either BF4 antibody (a, b) or FD4 antibody (e–f). In the absence of hormone, both immunoreactive chsp90 (a) or Δ B mutant (b) and the hMR (c, d) were mostly localized in the cytoplasm. After aldosterone treatment, immunoreactive hMR was clearly redistributed over the nuclei of infected cells (e), whereas hMR co-expressed with the Δ B mutant remained mostly cytoplasmic (f). (Magnification $\times$ 200.)

Table 3 Effect of the chsp90 mutants on the aldosterone-induced nuclear translocation of the hMR

High Five cells were infected by AcNPV-hMR together with AcNPV (control), AcNPV-chsp90 (chsp90), AcNPV- Δ A (Δ A), AcNPV- Δ B (Δ B) or AcNPV- Δ Z (Δ Z). The cells were incubated with 100 nM aldosterone for 16 h and processed for immunohistochemistry using the FD4 antibody to detect the nucleo-cytoplasmic distribution of the hMR. The positively stained cells were counted in 'blind' experiments by two independent observers and classified. Nuclear translocation (%) corresponds to the ratio between the cells presenting exclusively nuclear labelling and the total number of positive cells. Statistical significance: * $P < 0.001$ by the χ^2 test when compared with the control.

	Total number of positive cells	Nuclear translocation (%)
Control	816	27.4
chsp90	721	32.0
Δ A mutant	744	29.3
Δ B mutant	840	18.9*
Δ Z mutant	667	30.8

The nucleo-cytoplasmic distribution of the hMR co-expressed with each of the mutants was analysed after aldosterone exposure. As expected, aldosterone induced a nuclear localization of the hMR when co-expressed with the wild-type chsp90 (Figure 6e). In contrast, after aldosterone treatment, the hMR co-expressed with the Δ B mutant remained mostly in the cytoplasmic compartment, with few cells exhibiting nuclear staining (Figure 6f). This observation was confirmed by quantitative analysis. Comparison of the percentage of positive cells presenting exclusively nuclear labelling disclosed a significant difference ($P < 0.001$) between the nuclear translocation of the hMR with wild-type chsp90 compared with the Δ B mutant co-expression condition (Table 3). This result indicates a lack of hMR nuclear translocation, in accordance with the decrease in the hormone binding capacity of the Δ B-hMR complex already demonstrated *in vitro* (see Table 2).

DISCUSSION

In the present study we have demonstrated that recombinant steroid receptors (hMR and cER) are associated with co-expressed chsp90 in insect cells. The use of domain deletion mutants of chsp90 has led to the identification of two essential regions involved in steroid receptor function.

The baculovirus expression system is an efficient model for producing high levels of biologically active proteins. In this system the recombinant chsp90 displays the same physico-chemical characteristics as the avian oviduct protein [20], and the recombinant cER and hMR behave as authentic receptors in terms of high-affinity steroid binding, hetero-oligomeric structure and specific interactions with hormone response elements (present work; [35,40]). A scale-up of the baculovirus culture has provided us with sufficient quantities of steroid receptors and chsp90 to study protein-protein interactions, but we failed to demonstrate any *in vitro* reassociation of these proteins. Therefore we tested for *in vivo* association by co-expressing steroid receptors and chsp90 in the same baculovirus-infected cells. Under these conditions, the 8–9 S hetero-oligomeric complexes were displaced by BF4 antibody, indicating that the chsp90 is associated with the cER or the hMR, thus replacing the equivalent insect protein. The structure and ligand binding properties of the chsp90-steroid-receptor complexes did not seem to be affected by this protein substitution (insect hsp90/chsp90), consistent with the high degree of amino acid identity among hsp90 proteins from

different species [47]. The overexpression of chsp90 did not modify the amount of binding receptors, suggesting that hsp90 is not a limiting factor for the expression and functional assembly of the recombinant receptor complexes [35,48]. Indeed, it is known that other proteins participate to the assembly of functional hetero-complexes even in the absence of direct contacts with the receptor molecule.

The A region of hsp90 is involved in the interaction with the cER and hMR

All hsp90 proteins in eukaryotes contain a highly charged region in the N-terminal half of the protein [47]. This region, designated as the A region, is conserved among species with regard to its size, charge and location. Its secondary structure predicts an α helix, which may represent a surface feature likely to be a region of protein-protein interaction [34]. It has been proposed that this negatively charged region interacts with the positively charged segments located in the ligand binding domain of steroid receptors [31–33], and also with one segment of the DNA binding domain of the ER [33]. To further investigate this hypothesis, after co-expression of receptor and wild-type or mutant chsp90s, we analysed the cER or hMR co-immunoprecipitated proteins and showed that the Δ A mutant was not selectively linked to the receptor, consistent with a role for the A region in the hetero-oligomeric structure of the receptor. This A region has also been implicated in the interaction with the glucocorticosteroid receptor (GR) [49]. However, we cannot exclude the possibility that such a large deletion of 70 amino acids could modify the tertiary structure of the protein, leading in turn to an alteration of its interaction with the receptor outside the A region. A recent study using *in vitro* reassociation of a series of truncated and deleted chsp90s with the progesterone receptor showed that two regions (amino acids 381–441 and 601–677) of the chsp90 are critical for binding to the receptor [50]. On the whole, it is likely that hsp90-steroid-receptor interactions involve multisite contacts. Interestingly, HtpG, the *Escherichia coli* homologue of eukaryotic hsp90, lacks the equivalent A region [51]. GRs and MRs expressed in this prokaryote system are recovered as 4–5 S entities and/or with very reduced or no steroid binding ability [25,28,29], suggesting that they fail to interact with HtpG. This argument underlines the importance of the A region in the receptor-hsp90 interaction. We have shown a role for hsp90 in the maintenance of high-affinity hormone binding by the MR, in accordance to the results already obtained with the MR [28,29], the GR [25,52] and the dioxin receptor [53]. In contrast, progesterone [54] and androgen [55] receptors expressed in *E. coli* or the 4–5 S transformed hsp90-free ER [56] are still capable of binding their cognate ligands, illustrating a differential chaperone role for hsp90 among members of the steroid receptor superfamily.

The Δ B mutant compromises the hormone binding ability of the hMR but not of the cER

The B region of chsp90, representing amino acids 530–581, contains 29 charged residues out of 52 (56%). When the Δ B mutant was co-expressed with the hMR, the receptor lost its ability to bind aldosterone *in vitro*. This finding clearly indicates that the Δ B mutant is complexed with the receptor, confirming the results obtained with the immunoprecipitation assays, and strongly suggests that the B region of hsp90 is necessary to give a correct conformational structure to the ligand binding domain of the hMR. The modification of protein folding conferred by hsp90 binding has been already reported for MyoD1, which is also a DNA binding protein [42]. Moreover, immunohisto-

chemical studies extended our observation *in vivo*, since the ΔB mutant inhibited the aldosterone-induced nuclear translocation of the hMR. Our results present a clear demonstration that, *in vivo*, hsp90 can modulate the function of a steroid receptor. In contrast to the hMR, the binding of oestradiol by the cER was not affected by any of the chsp90 mutants, indicating that the ligand binding domain of the receptor is presumably correctly folded. In accordance with these results, association with a yeast hsp82 having a single amino acid substitution (Thr-525 to Ile), corresponding to Thr-541 of the chsp90 B region, led to the complete loss of MR signal transduction, whereas ER activity was almost unaffected [57]. This supports the notion that hsp90 has distinct roles with the different members of the steroid receptor superfamily. Note that, from the reported experiments, we did not find a specific role for the Z region of chsp90.

In summary, our results suggest that a critical and differential role is played by hsp90 in ER and MR function. The *in vivo* interaction of hsp90 with these steroid receptors requires the presence of the A region, although other parts of the hsp90 may also be involved. The B region is implicated in the ligand binding of the hMR, supporting the hypothesis that interaction with hsp90 is essential for the correct conformational structure of the receptor ligand binding domain.

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