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Are Receptor-Associated Nuclear Proteins Associated with the Earliest Effects of Steroid Hormones?

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The functional importance of the interaction of hsp90 with receptors for steroid hormones in the action of these hormones has been suggested. This hypothesis, although not yet proven, is supported by new data obtained in our laboratory and in those of others, whereas no conflicting experimental results have been presented. Our recent studies have dealt with the cloning of hsp90, transfection of normal and mutated receptors, the effects of the antihormone RU486 and immunohistochemistry.

Key words: Steroid receptors, Heatschok protein, RU 486.

This paper deals with very early steps in the action of steroid hormones (and antihormone), immediately after the binding of the hormone (or antihormone) to the receptor (R). We review data related to our current working hypothesis about the role of hsp90 in receptor function.

We are concerned with the receptors for estrogen (E), progestin (P), glucocorticosteroid (G), mineralocorticosteroid (M), and androgen (A). All these receptors belong to the *erb*-A superfamily (26), and thus they have the same overall structural organization (fig. 1). We suggest that they may mediate the activity of their corresponding hormones through a common mechanism.

Five years ago we presented the first data demonstrating the interaction between four steroid receptors (chick ER, PR, GR, and AR) (8) with a non-steroid binding protein of 90kDa. We chose to study the «non-transformed» 8S-form of steroid hormone receptors (8S-Rs), of calculated molecular weight of about 300,000 daltons (79) because we felt that it would

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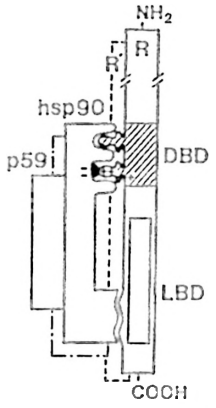

Fig. 1. *Diagrammatic representation of a steroid hormone receptor.*
The receptor itself includes a DNA-binding domain (DBD) and a ligand binding domain (LBD). In the case of ER, a second ER subunit (R') (66) is represented by a dotted frame. The difference in sizes of steroid hormone receptors is shown by the interrupted line in the N-terminal domain. Interaction with the heat-shock protein of about 90,000 daltons (hsp90) occurs at two sites, with LBD and DBD (the possibly ionic interaction between the positively charged amino acid of DBD with the negatively charged amino acids of the hsp90. A region is shown by + and −. The postulated dimeric state of hsp90 is indicated (−.−). The interaction between hsp90 and p59 (a nuclear protein of about 59,000 daltons) interaction is also represented.

be relatively easy to separate large, «non-transformed» steroid-binding complexes from other soluble proteins. The first evidence for the 8S form of steroid receptors was provided for ER by Toft *et al.* (82), and then generalized to other steroid receptors (7). 8S-Rs are recovered in the cytosol of target cells which have not been exposed to hormone, and have, thus, been envisioned as biologically inactive forms. These receptors, with a sedimentation coefficient in the 7S-10S range, have been described as oligomers (7, 79) or called «aggregates» (77). Up until 1983, no experimental data were available to describe the subunit composition of 8S-Rs. The purification of the 8S-PR from chick oviduct (69, 87), allowed to obtain a monoclonal antibody to be produced (62), and the subsequent demonstration of a 90kDa subunit common to the 8S-Rs for the five steroid hormones (8, 35, 41, 64). The work of Toft *et al.* led to a similar conclusion into respect to the chick PR (23). Soon thereafter, important observations were made: 1) the 90kDa protein and the 8S-Rs do not bind DNA, in contrast to the «transformed» receptor form into sedimentation coefficient of about 4S (4S-R, or 5S-R for the dimeric form of the ER), which binds both to non specific DNA and to hormone response element (HRE) DNA (11); 2) the 90kDa protein is a heat-shock protein of about 90,000 daltons (hsp90), as demonstrated by biochemical, immunological and cloning techniques (15, 16, 76, 78). Our working hypothesis, recently reviewed in detail (6, 9) is that hsp90 is responsible for the absence of binding to DNA by the 8S-Rs, and is released when the steroid hormone binds to the receptor, thus unmasking the DNA-binding site of the 4S-R. In the present paper, we present some new data from our laboratory which confirm and extend our original description of the heterooligomeric form of steroid receptors, and we discuss some controversial issues concerning the function of the 8S-Rs.

Steroid receptors and HSP90 are nuclear proteins. Do they associate physiologically to form «8S-receptors»?

It was known for some time that hormone-receptor complexes (H-Rs) are nuclear rather than cytoplasmic (24, 42), as «logically» expected for gene transactivators that bind to DNA (89), and this localization was confirmed by combined immunohistochemical and autoradiographic techniques (28). We then demonstrated that the chick oviduct PR is essentially located in target-cell nuclei, even

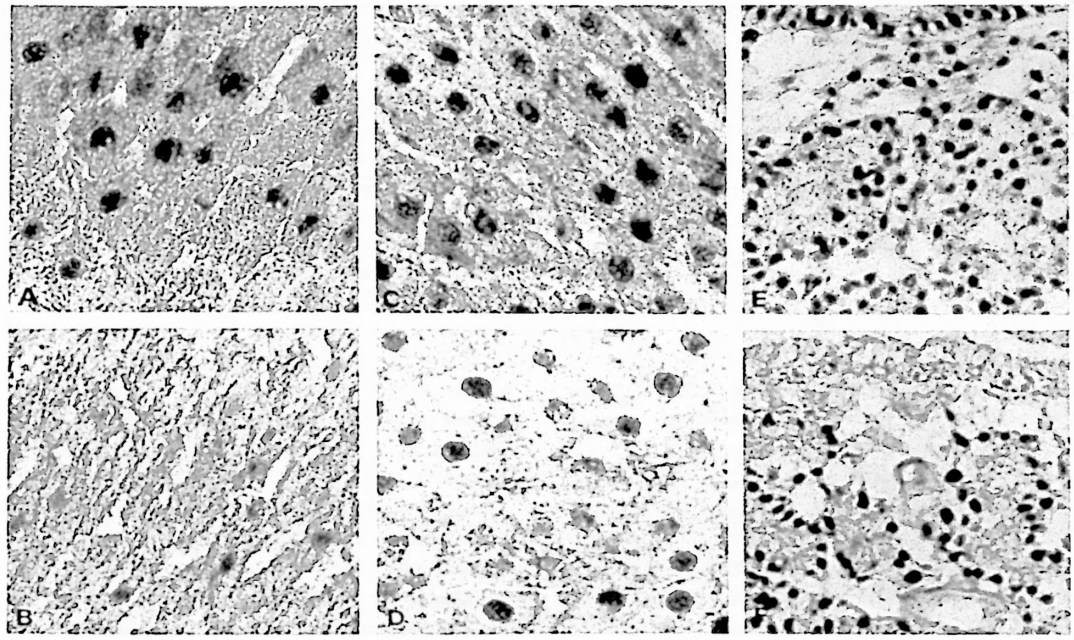

Fig. 2. *Glucocorticosteroid receptor: a nuclear protein.*
Comparison of the retention, in the nucleus, of hormone-free and hormone-occupied receptors. In frozen sections of the liver from a control and an Adx (adrenalectomized) rat (A), the immunostaining of the GR is completely lost if the section is pre-incubated in a buffered solution (PBS-sucrose) for 2.5 minutes before fixation (B). The same pre-incubation treatment has no effect on immunostaining of GR if the Adx rat has received dexamethasone (compare C, without, and D, with pre-incubation). The difference between B and D illustrates the low affinity of the binding of the hormone-free compared to the hormone-occupied nuclear GR. After treatment with dexamethasone, a higher affinity of the GR for nuclear structures allows better retention of receptors, i.e., an apparent increase, often interpreted as a result of nuclear translocation. Under the same conditions, in the chick oviduct, there is no substantial decrease in immunostaining of PR after pre-incubation in PBS-sucrose (compare E, without preincubation and F, with pre-incubation), although the immature chicken had not received progesterone. No increase in nuclear immunostaining of PR is observed after treatment with progesterone. Bar = 10 μm (27).

in the absence of hormone (28, 29), as was also found for the mammalian PR (57) and ER (43, 84). In the case of GR, a number of observations (reviewed in 37, 59) have indicated that it is mostly if not totally cytoplasmic prior to exposure of target cells to hormone. It has been suggested, furthermore, that the receptor is translocated to the nucleus upon exposure to hormone. We have found (fig. 2) (27) that GR is nuclear, but in the absence of hormone, it is not as firmly bound to the nucleus as ER and PR, a difference that could explain why GR is sometimes observed in the cytoplasm; nuclear GR may leak out of the nucleus during the fixation step which precedes immunocytochemistry. Thus, increased staining of the nucleus after exposure to glucocorticosteroid may be due to stronger nuclear attachment of the hormone-GR complexes, rather than to translocation of the GR from the cytoplasm to the nucleus. We have observed that the cytoplasmic GR-like antigen as detected

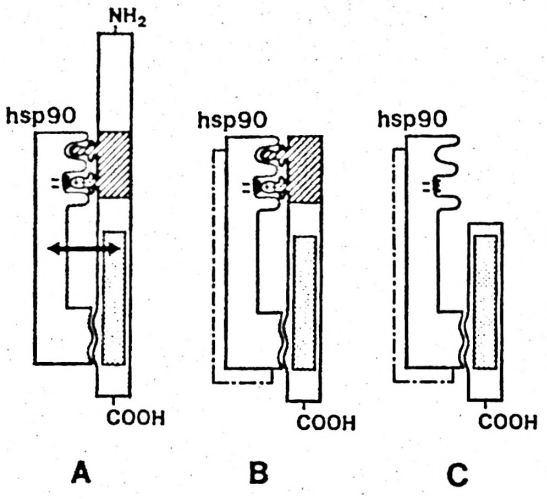

Fig. 3. *Diagrammatic representation of steroid receptors.*
A. «7S-PR» form (with one hsp90 molecule), obtained after purification, followed by cross-linking treatment with dimethyl pimelimidate (2). B. GR nt; mutant, lacking the N-terminal region (30). C. Trypsinized receptor, isolated after proteolysis of 8S-GR (cited in 21).

by immunohistochemistry, does not seem to participate in a translocation process when cells are exposed to hormone; the antibody may detect a variant of the GR in the cytoplasm, as for example GR-β which does not bind the steroid (39).

If, in absence of hormone, the receptors are nuclear and present in the 8S-R form (86), some hsp90, a cytoplasmic protein, should be demonstrably in the nucleus. However, it has been observed in nuclei of heat-stressed chick fibroblasts (18). Immunohistologically we detected the presence of hsp90 in the nucleus of all the cells we have examined in the chick (27, 29). Hsp90 is abundant from 0.5-2 % of soluble protein, depending on the tissue, and the presence of a small quantity in the nucleus would suffice to insure interaction with steroid Rs (concentrations of receptors are 100-1000 fold lower than that of hsp90). However, the mere presence of immunoreactive Rs and hsp90 in nuclei, while necessary for our hypothesis, does not prove the occurrence of 8S-Rs that contain hsp90.

Even if they are intrinsic nuclear proteins, 8S-Rs are found in the low-salt cytosol after homogenization of target cells which have not been exposed to hormone; they can also be demonstrated in the cytosol, at 0-4 °C, under isotonic conditions (e.g., in 0.15 MKCl) (4). The complexes are stabilized by oxyanions, molybdate ions (20), tungstate ions (55) or cross-linking agents (fig. 3A and 2, 72). It was found that the binding of hormone agonists, increased temperature, and/or increased ionic strength favor the 8S → 4S transformation (see reviews in 25, 52); the hormone dependent transformation of Rs from 8S → 4S forms occurs more rapidly as the temperature increases (21, 54). *In vitro* it is possible to transform the 8S-Rs into ~ 4S-Rs subunits, even in absence of

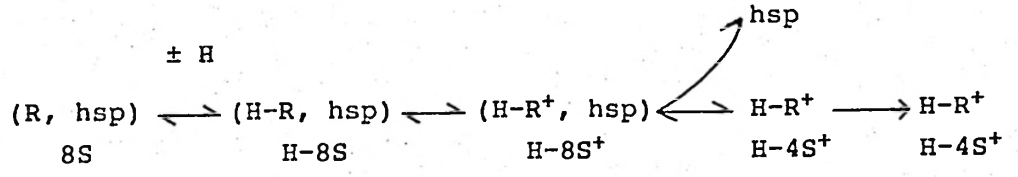

Fig. 4. *Series of reactions leading from the inactive, heterooligomeric 8S receptor to active hormone-receptor complexes.*
The stoichiometry of R and hsp90 in the 8S-R is not indicated (see text). The plus sign indicates that there is some activation of the receptor within the 8S complex (66). The plus sign indicates that the receptor is now in a form that is either biologically active or inactive, but in both cases it is unable to reassociate with hsp90.

hormone, by increasing the ionic strength (e.g., with 0.3-0.4 MKCl) at low temperature (90). *In vitro*, transformed receptors, whether devoid of ligand or bound to hormone or antihormone, can bind identically to HREs (5, 85). However, no direct demonstration that hormone-free 4S-R has biological activity has yet been provided. Indirect evidence strongly suggests that, in target cells, the hormone-4S-R complexes are biologically active (see review in 19, 88).

A number of studies favor the possibility that the 8S-R represents the physiological structure of receptors in target cells in the absence of hormone (49, 50), particularly studies in which the stabilizing effect of RU486 are exploited (47), protein labelling (40, 51), and cross-linking of proteins in intact cells (72). We believe that the hormone-induced dissociation of hsp90-receptor complexes is reversible (fig. 4), as has been shown with ER (73, 75 and REDEUILH, unpublished data). It may be more difficult to demonstrate this reversibility with the GR, which is highly unstable after the release of hsp90 (46). It is not known whether, after release of 4S-R from the 8S structure, there is further reversible or irreversible transformation related to the activity and/or inactivation of the receptor (fig. 4: *R). Furthermore, we interpret the constitutive activity of receptors devoid of the ligand binding domain (fig. 1) (38, 45) as an indication of hsp90-receptor interaction in cells (as discussed in the next section).

INTERACTION OF HSP90 WITH LIGAND-BINDING (LBD) AND DNA-BINDING (DBD) DOMAINS OF STEROID RECEPTORS

In all tested organs and species, cytosol 8S-Rs include at least one molecule of hsp90. The remarkable evolutionary conservation of hsp90s is demonstrated by comparison of nucleotide sequence data obtained from different species (13). (The results of experiments in which heterospecific receptors (human GR, human ER, and chick PR) were introduced into receptor-negative/poor cells (monkey, hamster and yeast, and human, respectively) (33, 34, 61 and CHAMBON, personal communication) suggest that transfected receptors bind endogenous and therefore heterologous hsp90s. This result provides the grounds for the suggestion that receptors for steroid hormones function via a common mechanism in all species.

In order to define which domain of steroid receptors is involved in the formation of complexes with hsp90, we performed transfection experiments using plasmids that encode wild-type and mutated GR and ER (38, 45). After transfection of GR-specific cDNAs that lack LBDs —(mutants 532* and 532-597 (38)— no 8S-GR was found, and the receptor was in a 4S-form (61). The same result was obtained with the HE15 and HE21 plasmids (45), which carries the gene for an estrogen receptor devoid of a steroid-binding region (CHAMBRAUD, unpublished data). We thus propose that there is binding of hsp90 to the LBD of normal GR and ER (fig. 1).

In the course of transfection experiments, some differences in hsp90-DBD interactions were observed when the GR and ER were compared. After transfection of cDNA that lacked DBD (61), the GR expressed in COS cells is in the 8S-form (the initially reported unstability is probably related to the increased susceptibility of mutated GR to proteolysis (CADEPOND and GROYER-SCHWEIZER, unpublished data)). Similarly, under the same conditions as those in which the GR-DBD appears to play a very minor role in the stability of 8S-R, we have observed that ER without DBD (HE4 in (45)) also is found in 8S form (CHAMBRAUD, unpublished data). Transfection experiments with mutated receptors only give indirect evidence for the regions involved and provide correlation with transcriptional activity. The

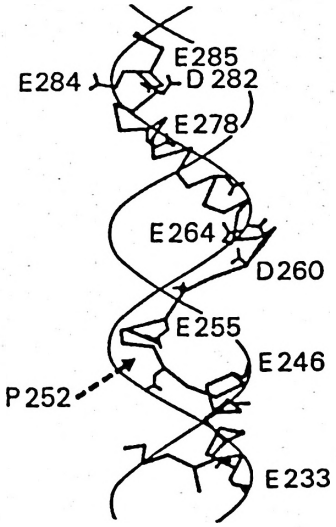

Fig. 5. *Sketely of the segment 228-290 of zone A of chick hsp90.*
The model of the segment was generated with the graphic system PS 390 of Evans & Sutherland and the MANOSK program. Segment 228-232, predicted with a low probability as helical, was modelled as a coil. Phi and psi angles of the loop region were manually adjusted in order to obtain the fitting of a maximum of negatively charged residues to the phosphate backbone (continuous double-helical line) of a B-DNA structure. During the fitting, the two α-helices (amino acids 233-247 and 261-287) were considered as rigid bodies. Only the α-carbon of the polypeptide backbone and the negatively charged side-chains are represented (chi angles of -50, 180, 180 and -50, 156 for Glu and Asp residues, respectively). The numbered residues are the nine most conserved negative amino acids between the five species; they are aligned with the DNA phosphate groups (13).

receptor may normally need a specific conformation to bind to hsp90. We also have observed that certain deletions in the DBD of GR can modify the steroid binding properties of the LBD (CADEPOND and GROYER-SCHWEIZER, unpublished data). It has been confirmed biochemically that GR-LBD can bind hsp90, since complexes containing LBD-hsp90 have been obtained after selective proteolysis of the 8S-GR (cited in (21)) (fig. 3C). Results of transfection experiments with GR and ER (to be published), as well as the demonstration of the 8S-form of the nt_i mutant of GR, which is devoid of the N-terminal end of GR, indicate that the N-terminal portion of receptors probably does not play an important role in formation of the 8S form (30) (fig. 3B).

Other data may be discussed in the context of interactions, between receptors and hsp90. Cloning and determination of the nucleotide sequence revealed a region A of 70 amino acids in chick and other hsp90s which is highly charged, with a predominance of negatively charged residues (13). Predictions of possible structures suggest two α-helices separated by a proline turn. Carboxyl groups of nine of the negatively charged amino acids, fully conserved throughout all species examined, can be aligned with the phosphate groups of a segment of a B-DNA double helix, as indicated by computer assisted, three-dimensional modeling (13, 48) (fig. 5). We propose that the A region interacts with the DBD of steroid receptors, particularly at the level of the positively charged amino acids present in the second Zn-finger and the C-terminal region of DBD. Experimental results are compatible with this hypothesis. An antibody raised against the A region of hsp90, which recognizes free hsp90, does not bind to hsp90 when it is included in 8S-PR (17). Similarly, an antibody against a sequence of amino acids which corresponds to the second finger of the chick PR, while able to bind to 4S-PR, does not bind to the 8S-PR (80). It is difficult to assess the part played by ionic bonds in the formation and stability of 8S-Rs, and there are probably differences between receptors (molybdate-stabilized 8S-ER dissociates more easily in a milieu of high ionic strength than does chick 8S-PR (67)). However, bonds other than ionic are probably involved and may obscure the electrostatic characteristics of interactions between DBD and hsp90.

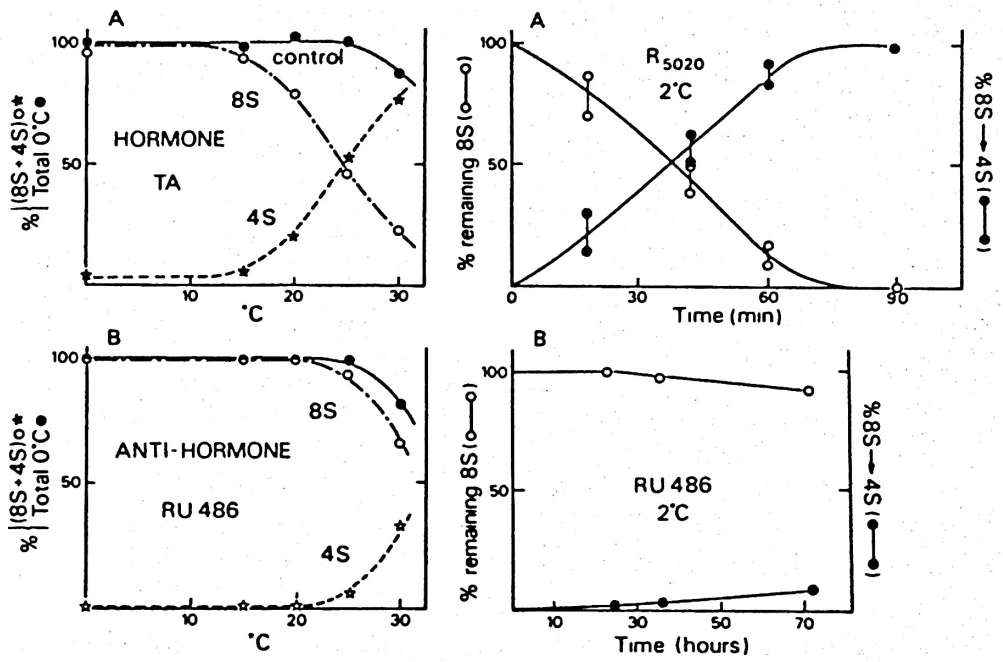

Fig. 6. *Effect of binding of RU 486 on transformations of 8S-GR (left) and 8S-PR (right) to their 4S-forms (B), compared to the effects of agonists (A).*
TA: triamcinolone acetonide; R_{5020}: progestin (36, 71).

HSP90 IN 8S-RECEPTORS: FUNCTIONAL ASPECTS

From the previous discussion, it appears that the role of hsp90 in receptor physiology could be to cap and thus mask the DBD of the receptor in its DNA non-binding 8S form. In the absence of hormone, hsp90 would be a negative regulator of DNA-binding receptors, since, in preventing binding of receptors to HREs, it would presumably preclude biological activity. We suggest that the attachment of hsp90 to the LBD is critical for the favorable positioning of hsp90, in order to direct the interaction of the A region of hsp90 with the DBD of the receptor. The remarkable correlation between the constitutive activity of truncated LBD-negative receptors (1, 32, 38, 45) with the absence of formation of the 8S-complex in transfection experiments (61 and CHAMBRAUD, in preparation) is an important argument in favor of our hypothesis. Binding of hsp90 to LBD could also explain 1. why chimeras of LBD and DNA-binding (Gal4 or LexA) or transcription-regulatory (E1A) elements (not all of the Zn-finger type) (31, 58, 83) are inactive in absence of hormone (the ionic interaction with hsp90 being, probably, relatively non-specific), and 2. how they are activated by the proper steroid ligand (activation would involved release of hsp90).

Pharmacological results obtained with RU486, an antiglucocorticosteroid and antiprogesterone steroid (10) are compatible with our model. When cytosol GR or PR binds RU486 instead of the corresponding agonist, the 8S → 4S transition is significantly impeded (fig. 6), even if the 8S → 4S transformation is stimulated by increasing the temperature or the concen-

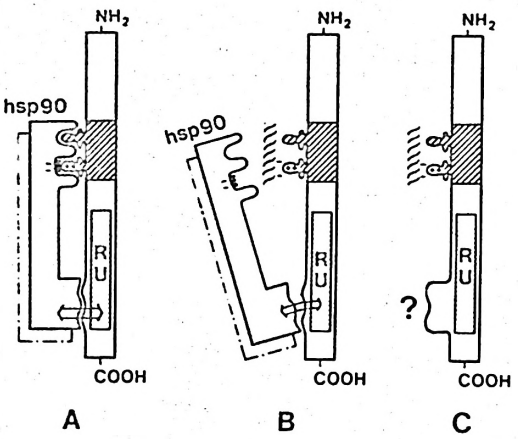

Fig. 7. *Mechanism of action of an antisteroid hormone at the receptor level: three possible models.* A. Stabilization of receptor-hsp90 interactions. B. Hsp90-LBD binding is maintained, but the DBD of the receptor is available for binding to HRE DNA (4,83). C. Release of hsp90, availability of the DBD for binding to DNA, and transconformation of the receptor, precluding any effect on transcription. RU: antisteroid hormone RU 486.

tration of KCl (36, 71). The difference between the kinetics of transformation of agonist-bound and antagonist-bound 8S-Rs may explain an aspect of the antagonistic activity of RU486, at the receptor level. DNA of the GRE in the TAT promoter is not protected from chemical methylation in HTC cells exposed to RU486 (12), or cultured in absence of glucocorticosteroid, in contrast to the protection apparently provided by agonist-receptor complexes. The lack of protection may be due to the DBD-capping activity of hsp90 (fig. 7A). It has recently been suggested that, in intact cells, RU486-GR (83) or RU486-PR (4) complexes, while being biologically inactive, can bind to the corresponding HRE. In figure 7B, we indicate that such a possibility does not exclude the hsp90-mediated mechanism of receptor function: binding of hsp90 to receptor may be conserved (or even reinforced), even if the antagonist-induced transconformation of LBD would lead to a weakening of hsp90-DBD interaction; the complex of hsp90-RU486-receptor would not, however, be able to induce transcription (because of interference with the function of transcription factors). We are aware that the stabilization of 8S-PR or 8S-GR by binding of RU486 may not be the only mechanism of action of the antihormone, and that other antihormones may act by a different mechanism; for example, binding of antihormone to LBD may induce a transconformation of the receptor (fig. 7C), which would impede efficient interaction of the receptor with other components of the gene transcription machinery, in contrast to the effect of an agonist.

In summary, capping of DBD, whether by ionic bonding or other interactions, and/or steric hindrance can be considered as a putative mechanism by which hsp90 could affect binding of receptor to HRE and consequently, the function of the receptor. Binding of hsp90 to LBD would permit the proper positioning of hsp90, preventing binding by the receptor to DNA. Hormone agonists would trigger the response, through binding to LBD, and the subsequent release of hsp90. Differences between agonistic and antagonistic functions may also be interpreted at this level.

Stoichiometry of the complex of receptor and associated proteins

Hsp90 is found as a dimer, whether isolated from the large pool of cytoplasmic hsp90 or released from purified 8S-R (63). We do not know whether or not hsp90 is a dimer within the 8S-PR or 8S-GR complexes, since experimentally only one molecule of hsp90-specific monoclonal antibody BF_4 can be bound to hsp90, regardless of whether the latter is free or included in 8S-Rs. Thus we cannot exclude the possibility that the 8S-R contains a single molecule of hsp90, which, upon release

from 8S-R, may combine with another hsp90 molecule to produce a dimer. Originally, we proposed that the 8S-PR (69) and 8S-GR (IDZIOREK, unpublished data) contain one molecule of receptor and two molecules of hsp90, and this proposal has been confirmed for the GR (22, 51). Our evidence for both 8S-GR and -PR is based on the densitometric scanning of electrophoretic gels and on the specific activity of purified 8S-R for chick PR, with one binding site per 250kDa, explainable by molecular weights of 100,000 daltons for the steroid-binding protein and 180,000 daltons for the non-steroid binding components (2 molecules of hsp90). The issue is different with the 8-9S ER (66, 75) since we demonstrated that it includes two molecules of ER in the hsp90-containing 8-9S form. That the original inactive form of PR and GR also include two molecules of ER in the hsp90-containing 8-9S form. That the original inactive forms of PR and GR also include two molecules of receptor and two of hsp90 is not excluded, since one molecule of receptor may be lost during the purification process, and the GR (56), like the ER (44, 74), may possibly be active in a dimeric form.

Another open question arises from the discovery that, in rabbit cytosol, a monoclonal antibody against a p59 protein interacts with 8S-PR and other 8S-Rs in the rabbit uterine cytosol (81). This observation has been extended (RENOIR *et al.* unpublished data) to several other mammalian (human, calf) cytosol receptors (the monoclonal antibody does not react with chick and rat proteins). We have not previously detected p59 after SDS-polyacrylamide gel electrophoresis of purified 8S-R, because, in the course of purification, there is a washing of the hormone-affinity column (68), which can remove p59 (unpublished data). Indeed, recent studies by immuno-affinity chromatography with an antibody against p59 have provided us with 8S-heterooligomeric receptors (rabbit PR and calf ER), which contain the p59 antigen in addition to hsp90 and the receptor itself (70). It has been also found that, unlike the 4S-receptors, hsp90 binds p59, in accordance with the model represented in figure 1. It has also been found that p59 is present in many types of cell, always in the nucleus (GASC, unpublished data).

TWO DIFFERENT FORMS OF NUCLEAR RECEPTOR

We suggest that there are two different forms of nuclear steroid receptors. In the absence of hormone, there is a *nuclear, non-transformed form of steroid receptor, which always includes hsp90 (probably in a dimeric form), the steroid-DNA-binding unit(s), and possibly p59* (in cells where it is expressed) (fig. 1). This nuclear 8S-form is inactive, but it is present in target cells prior to exposure to hormone; p59 may

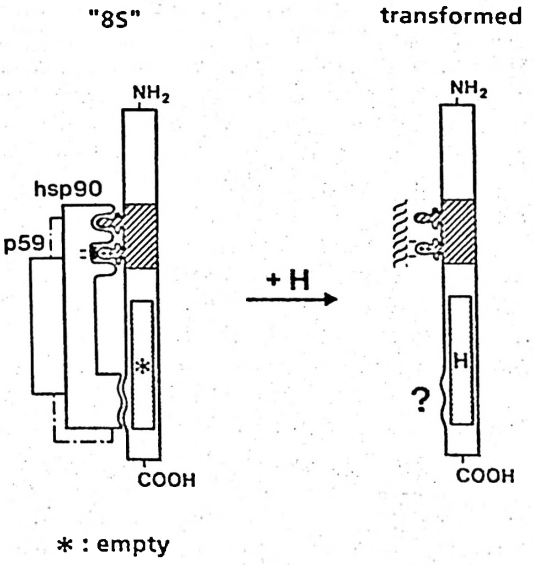

Fig. 8. *Transformation/activation of a steroid hormone receptor.*
The diagram represents the non-active 8S-receptor, as in fig. 1, and the release of the active hormone 4S-receptor complex upon binding of the hormone (H). Whether there is further transconformation of the «4S-R» is not known.

contribute to its nuclear location; it may be stabilized by binding of RU486 in the case of the GR and PR. It differs from the *nuclear, DNA-binding, transformed 4S form of the receptor* (fig. 8), which, after dissociation of the non-transformed receptor following binding of the agonist, is «activated», and is indeed active at the transcriptional level.

Two functions may be ascribed to the binding of hsp90 to steroid receptors. First, in the absence of ligand, hsp90 may contribute to the stabilization of the receptor protein: a number of experiments indicate a greater resistance of receptors to chemicals or increased temperature in their 8S-form, as opposed to their 4S-form (2, 14, 46). This protective effect may also explain the longer half-life of the receptor in the absence than in the presence of hormone (53, 65). Secondly, binding of hsp90 would provide repression of receptor activity in the absence of hormone. The conformational change provoked by steroid binding would trigger the response to the hormone by bringing about the release of hsp90, and thus the receptor would be able to bind the HRE, an interaction that probably leads to appropriate changes in the chromatin (3) and eventually, a transcriptional response.

Further studies are currently under way in order to verify our hypothesis and to explore the possible physiological, pharmacological and pathological consequences of our model. The hsp90 hypothesis may explain how the DBD of steroid receptors is hidden and does not function in the absence of hormone, and, conversely, how it becomes available for binding to DNA in a hormone-dependent manner. Hsp90 would function at the level of the DBD of receptor proteins. Correct positioning of hsp90, in order to cap the DBD, would be directed by binding of hsp90 to LBD. The LBD, which binds the steroid, would provide, presumably through a conformational change, the response to the hormone, with the release of hsp90. Hsp90 may thus provide an extramolecular mechanism involved in the control of the function of composite proteins, such as steroid receptors; the remarkable functional tolerance of the structure of steroid receptors to topological changes (26) is an important argument in favor of the hsp90 mechanism. Whether this abundant heat shock protein plays a similar role with other acidophilic proteins (including other transcription regulators) is unknown (however, interactions with a number of tyrosine protein kinases of viral oncogene origin, the eIF-2α kinase, and F-actin have been described (60)). It is proposed that, in the receptor molecule, LBD plays a signalling and transducing role, while DBD plays a functional (executive) role and hsp90 is an extrinsic repressor, released after binding of the hormone.

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Resumen

Se sugiere la importancia funcional de la interacción de hsp90 con los receptores para hormonas esteroideas en la acción de estas hormonas. Esta hipótesis, aunque todavía no demostrada, está apoyada por nuevos datos obtenidos en nuestro laboratorio y en otros, mientras que no se han presentado resultados contradictorios. Nuestros estudios recientes tratan de la clonización de hsp90, la transfección de receptores normales y mutados, los efectos de la antihormona RU486 y la inmunohistoquímica.

Palabras clave: Receptores esteroideos, Proteínas nucleares, Hormonas esteroideas.

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