The Functional Relevance of the Heteromeric Structure of Corticosteroid Receptors[a]

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Steroid receptors are transacting factors that must move through the cytoplasm to the nucleus to interact with the DNA. They are members of a superfamily with a well-known topology. The carboxy terminal region represents the ligand-binding domain and subsumes the specific recognition site for ligands. In the middle of the protein is the DNA-binding domain, which contains so-called cysteine-rich zinc fingers and is critical for DNA interactions. The amino terminal domain is thought to be important for transcriptional modulation. These domains appear to be functionally separable, and numerous chimeras have been constructed which retain appropriate binding selectivities and DNA recognition properties.[1,2] In this paper, we shall focus on two of the steroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), which bind the stress hormones cortisol/corticosterone and their analogs. They play a key role in the negative feedback mechanisms of the hypothalamic-pituitary-adrenal axis, and in the control of its circadian rhythm.

It is now generally accepted that the association of heat shock proteins (hsps) with the steroid receptors is important for the stabilization of the steroid-binding protein in a non-DNA-binding and transcriptional inactive form. This "repressor" function has been typically considered to be the main, if not the only, role of the associated proteins on the receptor function.[3] According to this view, GR would appear to be a unique steroid receptor, in that it additionally requires the association of hsps for adopting a high steroid-binding affinity state.[4–7] We recently found similar requirements for the MR.[8]

From a broader perspective, heat shock or stress proteins are considered to be part of the family of molecular chaperones. The members of this family were recently defined by Hendrick & Hartl[9] as "proteins that bind to and stabilize an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein, facilitate its correct folding *in vivo*: be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations." Within this context, the interaction between steroid-binding proteins and chaperones seems to illustrate only a particular example of the more general importance of chaperones in cell physiology. An interesting

[a]This work was supported by grants NIDA R01-DA02265, NIMH PO1-MH42251-06, and NIADDKD P30-AM34933.

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model supported by Pratt and his collaborators[10] basically conceives the steroid-free receptor complex as one of the products generated upon reaction of the steroid binding unit with a rather large chaperone complex. This heteromeric complex would contain hsp90, hsp70 and hsp56, and would be present in cytosol, independent of steroid receptors.

In systems of *in vitro* translation using reticulocyte lysate, and likely in situations *in vivo* as well, the association of the GR with hsps occurs as soon as or perhaps before the receptor is fully translated.[5] Subsequently, the potential of the lysate preparations in receptor assembly was successfully used to reconstitute previously transformed, hsp-free, receptors.[11,12] The feasibility of what can be regarded as a "reversal transformation" moved us to attempt the use of lysate for the *de novo* assembly of recombinant receptors generated in bacteria, a host that proved to be ineffective for the assembly of other steroid receptors.[8,13–17] We found that the bacterially expressed corticosteroid receptors MR and GR both reacted with lysate in a way similar to the *in vitro* transformed receptors, forming complexes including the 90-kDa heat shock protein, hsp90.[8,18] The resulting heteromeric complexes, unlike the uncomplexed receptors, closely resembled the native receptors in terms of their functional characteristics. However, the lysate untreated receptors evidenced some characteristics that differ from those expected for a typical transformed receptor. This result is difficult to fit within a model in which the only effect of the assembly is to prevent an intrinsically active receptor from being permanently active, without affecting its structure, and leads us to reconsider the potential role of hsps in the functioning of steroid receptors.

By combining the expression of recombinant corticosteroid receptors in bacteria and in eukaryotic cells, we recently learned more about the molecular and functional changes caused by receptor assembly. In this paper, we shall summarize our findings and try to interpret them within the context of other reports which, as a whole, point to a more general importance of the assembly process.

Expression of Corticosteroid Receptors in Bacteria

The DNA- and the steroid-binding domains of the rat MR and GR were expressed in *E. coli* as C-terminal fusion proteins of glutathione-S-transferase (GST) as previously reported for the rMR.[8] The subsequent biochemical characterization of both recombinant proteins was performed using two different bacterial preparations, referred to as crude and pure preparations, respectively. The crude preparations consisted of the supernatants resulting from the bacterial lysis and centrifugation. The pure preparations were obtained by subsequent affinity purification of the crude preparations in immobilized glutathione. For receptor assembly, the pure preparations were incubated with rabbit reticulocyte lysate,[8] resulting in the pure-treated preparations.

Characterization of MR and GR in Crude Preparations

As can be seen in TABLE 1, the crude preparations of MR and GR exhibited steroid-binding specificities that were consistent with those reported for the native receptors. For the MR, the potency of different steroids in [^{3}H]aldosterone competition-binding assays followed the order corticosterone ≥ deoxycorticosterone ≥ aldosterone > cortisol > spironolactone > progesterone > dexamethasone >>> RU26988/38486, testosterone or estradiol. For the GR, using [^{3}H]corticosterone, the

TABLE 1. Apparent K_i Values (nM) for Different Steroids[a]

Steroid	GR		MR	
	Crude	Pure-Treated	Crude	Pure-Treated
RU 28362	49 ± 1	1.9 ± 0.2	>1,000	>100
Dexamethasone	82 ± 7	2.2 ± 0.2	680 ± 106	20.2 ± 2.2
Corticosterone	122 ± 12	3.3 ± 0.1	20 ± 2	0.2 ± 0.1
Cortisol	208 ± 64	5.4 ± 0.2	95 ± 12	1.6 ± 0.2
Progesterone	453 ± 93	11.7 ± 1.2	500 ± 43	17.8 ± 1.8
Aldosterone	583 ± 99	17.4 ± 0.8	40 ± 5	0.6 ± 0.1
Deoxycorticosterone	600 ± 75	19.0 ± 1.0	23 ± 3	0.4 ± 0.1
Spironolactone	>1,000	>100	340 ± 19	6.5 ± 0.6
Testosterone	>1,000	>100	>1,000	>100
Estradiol	>1,000	>100	>1,000	>100

[a]At least three separate binding assays were performed with serial dilutions of the different steroid competitors (0.1–5,000 nM). The radioligand used was either [^{3}H]corticosterone (100 nM for crude GR and 5 nM for pure-treated GR preparations) or [^{3}H]aldosterone (50 nM for crude MR and 0.5 nM for pure-treated MR preparations).

rank order was RU28362 > dexamethasone ≥ corticosterone > cortisol > progesterone > aldosterone ≥ deoxycorticosterone >>>> spironolactone, testosterone or estradiol.

The reported apparent equilibrium dissociation constant (K_D, 4°C) values for corticosterone of native, cytosolic MR and GR approximately average 0.5 nM and 2.5 nM, respectively. However, the K_D values for the recombinant MR and GR in the crude preparations were significantly higher, implying a 78-fold and a 60-fold reduction in the steroid-binding affinity, respectively (see TABLE 2). The hydrodynamic characteristics of the receptors in crude preparations also differed from those observed in the native receptors. Sedimentation velocity analysis in sucrose density gradients of both recombinant MR and GR revealed that the steroid-bound

TABLE 2. Characteristics of the Bacterial Preparations of GR and MR[a]

Preparation	B_{max} (fmoles/mg Protein)	K_D (nM)	Sedimentation Coefficient (S)	Association with hsp90
GR				
Crude	443 ± 79	95 ± 28	≈ 4	–
Pure-treated	69,883 ± 9,884	3.8 ± 0.3	≈ 9	+
MR				
Crude	793 ± 158	47 ± 5	≈ 4	–
Pure-treated	21,700 ± 3,500	0.6 ± 0.1	≈ 9	+

[a]The binding capacity (B_{max}) and K_D values were determined by at least three separate Scatchard analyses using serial dilutions of [^{3}H]corticosterone for GR, and [^{3}H]aldosterone for MR. The sedimentation coefficient of the steroid-bound receptors was determined in linear sucrose gradients in the presence of molybdate. The presence of hsp90 in the receptor complexes was determined by the shift of the 9 S forms towards the bottom of the gradients when samples were incubated, prior to sedimentation, with an anti-hsp90 monoclonal antibody.

complexes migrated as 3.7–4.0 S species, as opposed to the typical 8.5–9.0 S species, hsp90-associated, observed in native receptor preparations. The presence in the gradients of molybdate or tungstate, which stabilize steroid receptors in the 9 S heteromeric forms, did not affect the sedimentation velocity of the recombinant receptors. This result strongly suggests that the steroid-binding protein does not interact with bacterial proteins or factors, at least in a manner stable enough to withstand the analytical procedure. Our results are in agreement with previous bacterial expression studies of glucocorticoid,[17] progesterone,[13–14] and androgen[15] receptors, which did not evidence the formation of definite heteromeric complexes upon sedimentation analysis. Interestingly, in these expression studies, the only receptor evidencing an abnormally low steroid-binding affinity was GR.[17] In addition, a similar low affinity was obtained for liver cytosolic GR preparations that had been previously activated to a DNA-binding, 4 S form.[16] On the basis of this similarity, it was postulated that the component(s) associated with the nonactivated GR, possibly the hsp90, would play an important role in stabilizing the receptor in a high-affinity state for steroids.[16] However, given that *in vitro* assembly of 9 S receptor complexes had proved unsuccessful at that time, the role of the associated components on receptor function could not be established.

In order to determine the DNA-binding characteristics of the steroid-receptor complexes generated in crude preparations, DNA-cellulose binding at 0°C was performed using the GR preparations. Interestingly, less than 10% of the radioactive complexes bound to the DNA-matrix. When samples were heated at 30°C for 1 h, or treated with 0.4 M KCl, conditions that typically promote receptor transformation, the percentage of the complexes bound to DNA did not significantly change.

Characterization of MR and GR in Pure Preparations

The incubation of affinity-purified preparations of MR and GR with concentrations up to 60 nM of different radiolabelled steroids, including [^{3}H]aldosterone, [^{3}H]corticosterone and [^{3}H]dexamethasone, resulted in negligible specific binding. However, after pretreatment of the preparations with rabbit reticulocyte lysate at 30°C to obtain "pure-treated" receptors, steroid-binding activity was detectable. This activating effect was gradual, and at 30°C the maximum binding was reached at 60 min and 30 min of treatment, for MR and GR, respectively. Incubation of the pure preparations alone at 30°C or in the presence of lysate at 0°C resulted in negligible steroid-binding activity. Besides the temperature dependence, the reaction with lysate also evidenced a strict Mg^{2+}/ATP dependence and best results were obtained when an ATP-regenerating system was used.[8] This time, in contrast with the crude receptor preparations, the observed K_D values (see TABLE 2) were in agreement with those reported for cytosolic receptors in rat brain[19–22] or for recombinant receptors expressed in eukaryotic cells.[23–26] The binding specificity pattern, evaluated as the potency rank order of different steroids in competition-binding assays, was the same as that observed with the crude preparations. However, the apparent inhibition equilibrium constant (K_i) values were at least one order of magnitude lower, reflecting the higher steroid-binding affinity of the receptors in the pure lysate-treated preparations. Interestingly, the sedimentation analysis of both lysate-treated preparations revealed that the steroid-receptor complexes migrate as approximately 9 S species in sucrose gradients containing 20 mM molybdate (TABLE 2). When molybdate was replaced by 0.4 M KCl, the sedimentation coefficient shifted to approximately 4 S, as occurs with native cytosolic receptors.

The presence of hsp90 in the steroid-receptor complexes was assessed by sed-

imentation-shift analysis[8] using the hsp90-reactive, 8D3 monoclonal antibody.[27] Only the 9S, and not the 4 S, complexes were shifted to 12–14 S species by preincubation of the samples with the antibody, indicating that hsp90 is a component of the 9.0 S forms of the lysate-treated recombinant MR and GR (TABLE 2).

DNA-cellulose binding revealed that both pure-treated complexes, MR and GR, can be activated to a DNA-binding form. Treatment of GR with 0.4 M KCl at 4°C for 30 min, or heating at 30°C for 1 h, significantly increased (~ 300%), over the control values, the fraction of receptor complexes bound to DNA. Treatment of MR with 100 mM thiocyanate, a chaotropic anion known to be effective for MR activation,[28] for 30 min at 30°C, or with 0.4 M KCl for 30 min at 4°C, also significantly increased the DNA retention values (79% and 37%, respectively) over the control values. In all the cases, the activating effect of the salt or temperature treatments was completely prevented when complexes were preformed in the presence of 20 mM molybdate.

Taken together, our results indicate that the reticulocyte lysate treatment of the pure receptor preparations promotes the *de novo* formation of heteromeric complexes comprising at least the steroid-binding protein and hsp90. Several lines of evidence support this conclusion:

a. The hydrodynamic behavior, the steroid-binding affinity, and the DNA-binding characteristics of the receptor complexes present in the pure lysate-treated preparations, which completely mimic the native receptors.

b. The reaction of the receptor complexes with the 8D3 monoclonal antibody against hsp90.

c. The temperature and energy dependence of the reaction between the pure preparations and the retyculocite lysate, which has also been observed in the reassociation of immunopurified progesterone and glucocorticoid receptors with heat shock proteins.[11–12,29]

Interestingly, the reassociation with heat shock proteins has only been observed when purified receptor preparations are used, the results being consistent with the presence of unknown inhibitory factors in crude cytosolic preparations. Our results are in agreement with this observation. Lysate treatment of crude bacterial preparations does not generate detectable amounts of hsp90-receptor complexes, although the nature of this inhibition is still unknown.

In addition to hsp90, other proteins, including hsp70 and hsp56, have been described to immunoprecipitate with the steroid-binding proteins of the different steroid receptors (for review, see Refs. 30–31). We are presently conducting experiments to determine the probable association of these proteins with the recombinant receptors during the lysate-mediated assembly.

The comparison between the hydrodynamic behavior, and the steroid- and DNA-binding characteristics of the receptors present in crude versus pure lysate-treated bacterial preparations strongly supports the important role of the assembly in receptor function. Our data show that the assembly process is necessary for the generation of corticosteroid receptor complexes with high affinity for the steroid. In agreement, rat epididymal sperm, which appears to lack hsp90, contains GR that fails to bind ligand.[32] In addition, our data show that the assembly process is required for the formation of receptor forms capable of undergoing transformation to a DNA-binding state. This last requirement may explain why the glucocorticoid receptor, when expressed in a strain of yeast with limited expression of hsp, is ineffective in promoting transcriptional activation from a glucocorticoid-responsive reporter plasmid.[33] The results in yeast constituted the first *in vivo* suggestion of the implication of hsp90 in the process of receptor activation. However, given that hsp90 is essential for yeast survival, the expression of hsp90 was not completely abolished and only

a decrease in the ratio of hsp90 to receptor levels could be achieved. As opposed to yeast hsp90, the bacterial hsp90 homologue C62.5[34] does not appear to form stable complexes with corticosteroid receptors.[8,13–17] This difference could reflect the fact that the predicted amino acid sequence of C62.5 is only 42% identical to human hsp90, and also lacks a fifty amino acid stretch which is believed to be important for interaction with steroid receptors.[35]

In agreement with the *in vivo* evidence, our results with the crude preparations suggest a key role of hsps in the appropriate folding and transformation of the receptors and is at odds with a model in which hsp90 is seen primarily as a mechanism for preventing the steroid-binding protein from interacting with target DNA as proposed by Baulieu *et al.*[3]

Expression of the Glucocorticoid Receptor in Mammalian Cells

In order to further understand the structural requirements for the *in vivo* GR function, mutant GRs were expressed as the rat full-length receptors in mammalian Cos-1 cells. For comparison, the same mutants, lacking the N-terminal region, were also expressed in bacteria as fusion proteins and treated with reticulocyte lysate as described above. Two mutants were designed with substitutions in the hsp90-binding region of GR[36] (see FIG. 1). In the GRO mutant, Pro 643, located in a conserved hydrophobic stretch of the hsp90-binding region, was replaced by Ala. In the GR8, two substitutions were made in the conserved N-terminal end of the hsp90-binding region, which consisted of the replacement of Lys 597 by Ile, and Pro 600 by Leu.

In both expression systems tested, the GRO mutant exhibited a significant 4-fold reduction in steroid-binding affinity, in comparison with the wild type control. Interestingly, the binding specificity of GRO for different steroids did not change in either of the assayed systems. Since the steroid-binding activity of the GR8 mutant was substantially reduced in both systems, an accurate binding characterization could not be performed with this mutant.

Differences between the GRO mutant and the wild type GR were also detected in the sucrose-gradient sedimentation characteristics. In the presence of molybdate, under conditions in which the steroid-bound complexes of GR migrated exclusively as 9 S forms, more than 50% of the GRO complexes migrated as smaller, 4 S forms. This result was identical in both expression systems (mammalian and bacterial) and strongly suggests that the stability of the hsp90-containing 9 S forms is reduced in the GRO mutant. Moreover, under activating conditions, both receptors, GR and GRO, migrated exclusively as 4 S forms.

In order to evaluate the possible functional implications of the structural changes in the GRO mutant, transcriptional activation assays were conducted. Interestingly, when a glucocorticoid-responsive reporter plasmid was used, the GRO mutant showed only 30% of the hormonal response of the wild type GR. This decrease was observed even at maximal concentrations of ligand, eliminating the differences due to affinity changes; furthermore, the decreased transcriptional activation was seen after correcting for levels of expression and binding (B_{max}), indicating that the loss cannot be simply explained on the basis of diminished receptor protein, decreased ligand availability or decreased binding. In summary, in comparison with the wild GR, GRO exhibited: (i) normal, full *in vitro* dissociation from hsp90 under activating conditions, (ii) reduced but not suppressed steroid- and hsp90-binding, and (iii) reduced transcriptional activation. These facts lead us to suggest that proper receptor-hsp90 interaction, and/or stability of the heteromeric form of the receptor as a whole, are required for the transcriptional activation process. The chaperone role of

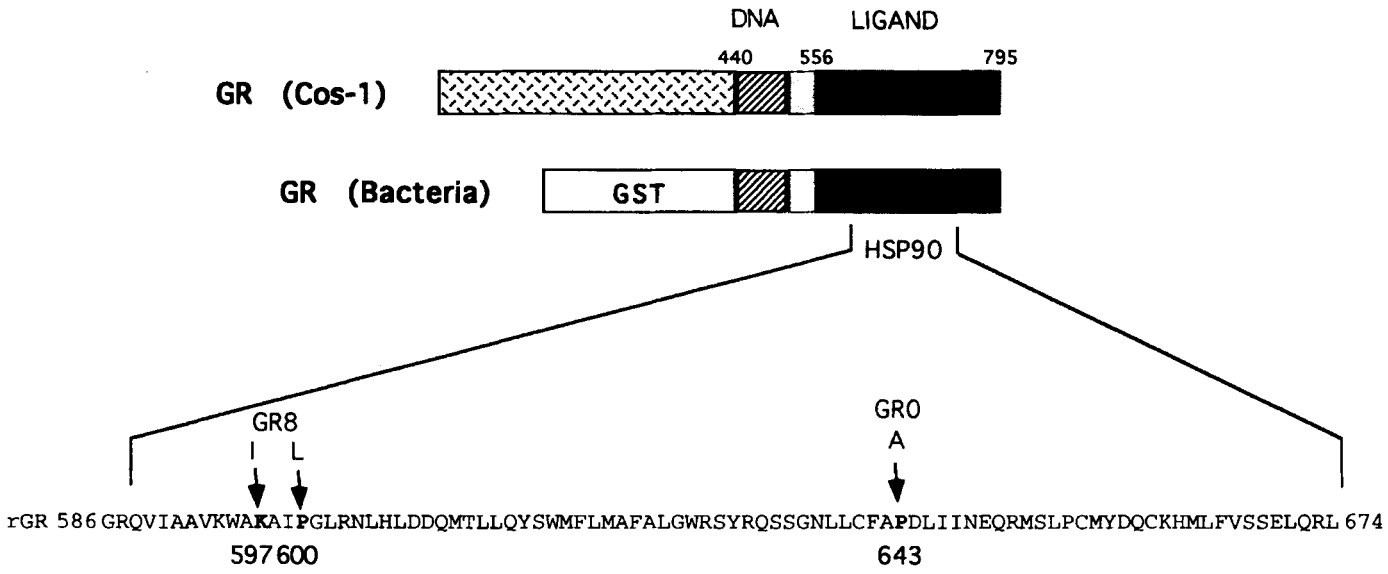

FIGURE 1. Schematic representation of the GR mutants generated in Cos-1 cells and in bacteria. GST, glutathione-S-transferase; DNA-, ligand-, and hsp90-binding domains of the receptor. The numbers below the amino acid sequence correspond to the rat GR sequence.

hsp90[37,38] supports the conclusion that the mutation might alter the hormonally controlled switching between the transcriptionally active/inactive conformations of the receptor complex. Consequently, the conserved hydrophobic region in GR, involving the Pro 643, might be a key mediator in receptor transformation.

It has been shown that mutant steroid receptors that do not associate with hsp90 are constitutive activators of transcription.[39–41] On this basis, an "inactivation" function was postulated for hsp90. In mutational analysis, however, the complete steroid-binding domain had to be deleted in order to completely abolish the hsp90 association. Importantly, the same receptor domain has been reported to influence the intracellular solubility of overexpressed receptors.[42]

Our findings are in accordance with these latter observations, and provide experimental support to an emerging model in which hsp90 association, or the assembly in general, are required to permit, and not to impede, function of the full-length, wild type receptor.

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DISCUSSION OF THE PAPER

D. B. DEFRANCO (*University of Pittsburgh*): Is the assumption you are making that hsp-90 has no nuclear function?

AKIL: It is not something that I have any direct opinion about. It is the model, and the model may be incorrect. I absolutely agree with you that the conformation change may not lead to the shedding of hsp-90. What do you think?

DEFRANCO: I think what your data suggest is that there might, in fact, be some role for hsp-90 in the nucleus.

AKIL: The main reason I am showing the data in this rather physiological meeting is that there is a lot more to the molecular interactions. But whether the receptor acts through heat shock proteins or through folding and interaction with other proteins or directly, I do not know.

Note: The following discussion focused on some data showed by Dr. Akil regarding the existence of multiple MR isoforms, with different 5'untranslated regions, called α, β, γ, which have differential expression in brain regions and across development. This aspect is not covered in her paper.