BCR/LS 4
The Steroid/Thyroid Hormone Receptor
Family and Gene Regulation

THE ASSOCIATION OF THE GLUCOCORTICOID RECEPTOR WITH M_r 90,000 HEAT SHOCK PROTEIN AND TUBULIN.

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SUMMARY: The rat liver glucocorticoid receptor (GR) is associated with an M_r ~ 90 000 heat shock protein (hsp90) which *in vitro* has been shown to affect the functional status of GR. When GR is associated with a dimer of hsp90 it is unable to bind to DNA. By a ligand- and temperature-dependent process GR dissociates from hsp90 and becomes functionally active, in terms of capacity to bind to specific and non-specific DNA-sequences. Hsp90 interaction with GR has been shown to require at least the presence of an intact steroid binding domain of GR. After *in vitro* expression of rat GR in a reticulocyte lysate system GR interacts with rabbit hsp90 preexisting in the lysate.

By immunocytochemical staining of normal human fibroblasts with antibodies monospecific for GR, hsp90 and tubulin it has been possible to show a colocalization of these three proteins in the cell cytoplasm. After treatment with microtubule depolymerizing substances, e.g. colchicine, tubulin staining is redistributed, whereas other cytoskeletal proteins are unaffected by the treatment. GR and hsp90 immunostaining remains colocalized with tubulin-staining also after this drug treatment.

INTRODUCTION

Glucocorticoids, like all steroid hormones, exert their biological effects via a specific intracellular, soluble receptor (Yamamoto, K.R., and Alberts, B.M., 1976). The glucocorticoid receptor (GR) is present in cytosol in a non-DNA-binding form, commonly called non-activated receptor (or non-transformed receptor). Binding of the steroid to its receptor causes a change

in the receptor's ability to bind to DNA. Interaction of the receptor with specific target elements on the DNA, i.e. glucocorticoid response elements (GREs), modulates the transcription of specific genes (Ringold, G.M., 1985; Yamamoto, K.R., 1985; Gustafsson, J.-Å. et al., 1987).

Cytosolic non-activated glucocorticoid receptor from target cells has a sedimentation coefficient of ~9 S and a Stokes radius of 7-8 nm, which corresponds to an oligomeric structure with a molecular weight of ~300,000 under non-denaturing conditions (Sherman, M.R., and Stevens, J. 1984; Holbrook, N.J., et al., 1985). The activated GR has an M_r ~ 100,000 (sedimentation coefficient of ~4 S, Stokes radius of 5-6 nm). Analysis under denaturing conditions have shown that the glucocorticoid-binding entity is an $M_r \approx 94,000$ protein in mammalian cells in general (Carlstedt-Duke, J. et al., 1987; Brönnegård, M. et al., 1987; Gronemeyer, H., and Govindan, M.V., 1986).

The large 9 S complex can be observed when analysis is performed in the presence of molybdate, or other transition metal oxyanions from group VI-A (Leach, K.L, et al., 1979; Dahmer, M.K. et al., 1984). Molybdate has been useful in characterization and purification studies because it stabilizes the non-activated glucocorticoid receptor. The ~ 9S GR-complex observed in vitro has been suspected to represent artifactual association of several subunits depending on the presence of molybdate. However, the large complex is also detectable under molybdate-free conditions (Lapointe, M.C. et al., 1986; Denis, M. et al., 1988a; Denis, M. et al., 1988b), i.e. in hypotonic buffers. Analysis of the molybdate-stabilized complex, using a monoclonal antibody raised against the activated glucocorticoid receptor from rat liver (Okret, S. et al., 1984), has shown that the complex contains a single steroid-binding subunit recognized by the antibody (Okret, S. et al., 1985).

Analysis of the molybdate-stabilized glucocorticoid receptor, purified by steroid affinity chromatography or immunoaffinity chromatography, has shown that the complex contains an M_r ~ 90,000 non-steroid-binding protein (Housley, P.R. et al., 1985; Mendel, D.B. et al., 1986). This protein corresponds to the M_r ~ 90,000 heat shock protein, hsp90 (Sanchez, E.R. et al., 1987; Catelli, M.G. et al., 1985).

We have been able to purify to near homogeneity the rat liver glucocorticoid receptor-associated hsp90 by using a combination of immunoaffinity chromatography and high performance anion-exchange chromatography (Denis, M. et al., 1987). We also performed a characterization

of the hydrodynamic parameters of the purified proteins. Based on the calculated molecular weight of the hsp90 and of the different receptor forms (Table 1), we, in analogy to others (Mendel, D.B., and Orti, E., 1988) have proposed that the 9 S, non-activated glucocorticoid receptor might be composed of one M_r ~ 94,000 steroid-binding unit and a dimer of hsp90 (Denis, M. et al., 1988c).

The purified rat glucocorticoid receptor-associated hsp90 was used as antigen, in order to raise antibodies in rabbits (Denis, M. 1988d). Following affinity purification of the antibodies on hsp90 coupled to either nitrocellulose or cyanogen- bromide- activated Sepharose 4B, a monospecific recognition of a single M_r ~ 90,000 protein in rat liver (Denis, M., 1988d) on Western immunoblots was obtained. The antibodies were also reactive with the molybdate-stabilized 9 S glucocorticoid-receptor complex but not with the 4 S glucocorticoid receptor monomer, as assessed by sedimentation shift analysis on sucrose gradients.

A high-yield, efficient purification technique of hsp90 was developed, combining high performance anion-exchange and size-exclusion chromatography (Denis, M., 1988d). The monospecific anti-hsp90 antibodies were used to follow and evaluate this purification procedure. The N-terminal amino acid sequence of the purified protein (hsp90) was determined by automated Edman degradation. The 25 N-terminal residues detected showed a strong similarity with hsp90 from other species (Denis, M., 1988d).

How the domain(s) of the glucocorticoid receptor interact(s) with the hsp90 has not been fully elucidated. However, studies with GR mutants lacking the N-terminal domain (Gehring, U., and Arndt, H., 1985), as well as studies where the GR N-terminus has been removed by endogenous (Mendel, D.B. et al., 1985) or exogenous proteases (Gehring, U., and Arndt, H., 1985) indicate that the N-terminal so-called immunogenic GR domain (Carlstedt-Duke, J. et al., 1982) is not required for the interaction of GR with hsp90.

In experiments with COS-7 cells transfected with mutants of the human glucocorticoid receptor cDNA (28) the sedimentation behaviour of the expressed receptor has been analyzed. Only when a large part of the steroid-binding domain, residues 532-697 (corresponding to resídues 550-715 of the rat GR was deleted, the mutant receptor was found as an ~ 4 S entity (as opposed to ~8-9 S) even in the presence of molybdate (Pratt, W.B. et al., 1988). This was in contrast to when the DNA binding domain was changed, which

did not lead to any loss of hsp90 binding ability. These experiments would indicate that the steroid-binding domain is required for maintenance of the 9 S heteromeric complex. In this context, it was also postulated that a sequence of approximately 20 amino acids, localized in the steroid binding domain, which is conserved among the members of the steroid hormone receptor family, would be essential for the hsp90 interaction.

Molybdate stabilizes the glucocorticoid receptor in a non-DNA-binding form (Dahmer, M.K. et al., 1984). The dissociation of hsp90 is a prerequisite for DNA-binding of the M_r ~ 94,000 receptor monomer (Housley, P.R. et al., 1985; Mendel, D.B. et al., 1986). Activation to a DNA-binding state in vitro, can be induced by different manipulations, e.g. heat-treatment (Milgrom, E., 1981; Schmidt, T.J., and Litwack, G., 1982), and has been proposed to represent a hormone-dependent event (Schmidt, T.J., and Litwack, G., 1982; Moudgil, V.K. et al., 1986; Groyer, A. et al., 1986; Becker, P.B. et al., 1986; Sanchez, E.R. et al., 1987), although this issue has recently been challenged (Willmann, T., and Beato, M., 1986).

RESULTS AND DISCUSSION

Hsp90 interacts with the steroid-binding domain of rat GR: Based on the fact that trypsin treatment has been shown to degrade both crude and purified preparations of glucocorticoid receptor to an $M_r \approx 27,000$ fragment which binds steroid (Wrange, Ö., and Gustafsson, J.-Å., 1978; Carlstedt-Duke, J. et al., 1982; Wrange, Ö. et al., 1984), we analyzed the domain dependence of GR-hsp90 interaction. We investigated the effect of trypsin on the molybdate-stabilized glucocorticoid-receptor complex (Denis, M., Gustafsson, J.-Å. and Wikström, A.-C., in press, J. Biol. Chem.) (Table I). Also in the presence of molybdate, limited trypsinization (36) generated an $M_r \approx 27,000$ steroid-binding fragment.

However, this was not reflected in major changes in the tested physicochemical parameters of the trypsin-treated glucocorticoid-receptor complexes as compared to those in crude cytosol (Table I).

Table I Characterization of the hsp90 and the glucocorticoid receptor from rat liver

Characteristics were taken from Okret, et al., 1985, Denis, et al., 1987 and Denis et al., 1988c, or unpublished data.

	hsp90	Glucocorticoid Receptor			
		+ Molybdate		- Molybdate	
Trypsin treatment		-	+	-	+
Elution from Mono Q (M NaCl)	~0.36	~0.32	~0.35	~0.10	~0.04
R_s (nm)	~6.9	~7.4	~6.4	~5.5	~2.5
$s_{20,w}$ (S)	~6.1	~9.2	~8.3	~4.3	~2.7
Native M_r	~180,000	~290,000	~220,000	~100,000	~30,000
M_r from SDS-PAGE	90,000	94,000	27,000	94,000	27,000
Form	Dimer	Heteromer	Heteromer	Monomer	Monomer
Precipitation with anti-hsp90 antibodies	+	+	+	-	-
Binding to DNA	-	-	-	+	-

Furthermore, this $M_r \approx 27,000$ steroid-binding fragment could be immunoprecipitated by anti-hsp90 antibodies. This means that the C-terminal third of the receptor protein (residues 518-795 for the rat receptor) which contains the ligand-binding domain (Giguère, V. et al., 1986; Godowski, P.J. et al., 1987; Rusconi, S., and Yamamoto, K.R., 1987) by itself is sufficient to allow an interaction with hsp90.

Thermally induced conversion of GR to a DNA-binding state is a hormone-dependent event: The effect of exposure to hormone and/or to elevated temperature on the conversion of the glucocorticoid receptor to its 4 S form was investigated. A shift in sedimentation position from 9 S to 4 S of the glucocorticoid receptor only occurred when incubation with hormone was performed prior to heat-treatment (Denis, M. et al., 1988b). The position of GR in the gradient was determined independently of its steroid-binding capacity, by an enzyme-linked immunosorbent assay based on monoclonal and

polyclonal antibodies raised against the rat liver glucocorticoid receptor (Denis, M. et al., 1988b).

The effect of different manipulations such as heat treatment and addition of ligand on the DNA-binding properties of the glucocorticoid receptor was also tested. Following treatment, samples of rat liver cytosol were incubated with a radiolabelled specific DNA-fragment containing three glucocorticoid receptor-binding sites. The glucocorticoid receptors present in the samples were then immunoprecipitated by means of a monoclonal receptor antibody coupled to CnBr-Sepharose 4B (Denis, M. et al., 1987). The monoclonal antibody used (N^{O} 7, Okret, S. et al., 1984) recognizes the receptor in its monomeric DNA-binding (Okret, S. et al., 1984), molybdate-stabilized non-activated (Denis, M. et al., 1987), and unliganded (Wikström, A.-C. et al., 1987) forms.

Only labelling of cytosol followed by heat-treatment was found to result in co-precipitation of labelled DNA together with the receptor (Denis, M. et al., 1988b). This corresponds to an observed reduction of both the size and the total negative charge of the glucocorticoid receptor, as assessed by the glucocorticoid behaviour on high-performance anion-exchange chromatography (Denis, M. et al., 1988a). In conclusion, the heat-induced activation of the glucocorticoid receptor to a specific DNA-binding state is inducible by the binding of ligand. These results are summarized in Table II.

Association with hsp90 in cell-free lysates has been shown to occur for the steroid hormone receptors in general (Catelli, M.G. et al., 1985; Joab, I. et al., 1984), the progesterone receptor (Dougherty, J.J. et al., 1984; Renoir, J.-M. et al., 1984; Renoir, J.-M. et al., 1986) and the estrogen receptor (Redeuilh, G. et al., 1987; Sabbah, M. et al., 1987) in particular. The receptor for the steroid pheromone antheridiol from the water mold Achlya ambisexualis also associates with hsp90 (Riehl, R.M., et al., 1985).

Molecular cloning of the vitamin D (Baker, A.R. et al., 1988), thyroid hormone (Weinberger, C. et al., 1986; Sap, J. et al., 1986) and retinoic acid (Petkovic, M. et al., 1987; Giguère, V. et al., 1987; Benbrook, D. et al., 1988) receptors has demonstrated a high degree of similarity with steroid hormone receptors, especially in their DNA-binding region. Interestingly, the domain which has been proposed to interact with hsp90 (Pratt, W.B. et al., 1988) is also fairly well conserved, although these receptors have not yet been shown to interact with hsp90. Thus, it is possible that the receptors

Table II Effect of hormone-binding and heat-treatment on the DNA-binding ability and the sedimentation coefficient of the glucocorticoid receptor

Data are taken from Denis, et al., 1988b.

Treatment	Specific DNA-binding	Sedimentation Coefficient	Elution from Mono Q
No treatment	-	~9 S	~0.3 M
Labelling with hormone	-	~9 S	~0.3 M
Labelling with hormone then heat treatment	+	~4 S	~0.1 M
Heat treatment	-	~9 S	~0.3 M
Heat treatment then labelling with hormone	-	~9 S	~0.3 M

for steroid hormones, thyroid hormones, retinoic acid and possibly dioxin belong to a superfamily of receptors which contain, in addition to a binding site for specific DNA-sequences, a region that encompasses a putative domain of interaction with hsp90.

Translation of glucocorticoid receptor mRNA in vitro yields a non-activated protein: The interaction between glucocorticoid receptor and hsp90 has mainly been demonstrated in cell-free lysates. Only recently there is a report indicating that this interaction occurs also in vivo, as shown by in vivo pulse chase labeling of GR and hsp90 (Howard, K.J., and Distelhorst, C.W., 1988). To further elucidate the interaction of GR and hsp90 we have performed in vitro translation experiments in rabbit reticulocyte lysates (Denis, M. and Gustafsson, J.-Å., submitted for publication). By Western immunoblotting experiments, we demonstrated the presence of hsp90 in the reticulocyte lysate. GR synthesized under cell-free conditions in the lysate interacts with the preformed hsp90 both in the presence and absence of ligand, as demonstrated by sucrose gradient centrifugation (Table III). This in vitro synthesized GR can be converted to a DNA-cellulose binding form following labeling with dexamethasone and heat treatment (Table III).

Table III

	Binding to DNA cellulose % of added material		Sedimentation coefficient on 5-20% sucrose gradients
	Experiment no 1	2	Experiment no 1 and 2
Untreated lysate (+ Molybdate)	7.4%	6.6%	8S
Labelling with hormone and heat treatment	35%	32.5%	4S

The results from these experiments suggest that the occurrence of the nonactivated GR in 9 S complexes in cytosol is not merely an artifact due to cell homogenization. Furthermore, previous difficulties to reassociate GR and hsp90 after dissociation in vitro may be explained either by irreversible conformational changes of GR taking place after activation or by the requirement of a specific GR conformation occurring during or just after synthesis.

Immunocytochemical colocalization of GR with cytoplasmic microtubules and hsp90 in normal human fibroblasts: Previous immunohistochemical studies have revealed that hsp90 is predominantly localized in the cytoplasm of the cell (Lai, B.T. et al., 1984; Carbajal, M.E. et al., 1986; Gasc, J.-M., and Baulieu, E.E.; 1987; Koyasu, S. et al., 1986), where it could be associated with actin (Koyasu, S. et al., 1986; Nishida, E. et al., 1986). We have recently studied the intracellular localization of hsp 90 and GR in human fibroblasts (Akner, G., Sundqvist, K.-G., Denis, M., Wikström, A.-C. and Gustafsson, J.-Å., submitted for publication). In these studies we find an apparent co-localization of GR and hsp90, predominantly in the cellular cytoplasm. However, there is also a slight staining for hsp90 in the fibroblast nuclei, as determined by optical sectioning by confocal laser microscopy. The presence of hsp90 in the cellular nucleus would be a

prerequisite for a postulated *in vivo* role of hsp90 for steroid receptors that are supposed to reside in the nucleus, even in the absence of steroid (Perrot-Applanat, M. et al., 1985; King, W.J., and Greene, G.L., 1984; Welshons, W.V. et al., 1984; Gasc, J.M., and Baulieu, E.E., 1986). Since hsp90 represents 1-2% of the total cellular protein in most cells (Denis, M., 1988d; Lai, B.T. et al., 1984), only a small portion of it might be sufficient to allow the formation of receptor-hsp90 complexes, of unliganded receptors (ie. ER and PR) also in the cell nucleus.

In previous studies (Wikström, A.-C. et al, 1987) we noticed that GR immunostaining in a human uterus carcinoma cell line, NHIK 3025, apart from a diffuse cytoplasmic stain also seemed to be more intense in a fibrillar pattern well corresponding to the pattern of microtubules (Fig. 1).

At this stage, it was not possible to exclude that the fibrillae might correspond to actin or intermediary filaments. By treating normal human fibroblasts with microtubule disrupting drugs such as colchicine, at a concentration of 10^{-5} M, for 60 min, it was possible to show a concomittant redistribution of GR and tubulin immunostaining in double-staining experiments. The pattern of actin and vimentin staining in these cells was not affected in a similar fashion. Further studies of this possible tubulin-GR interaction are now in progress.

In conclusion, all the data described here are consistent with the model of an *in vitro* activation of the glucocorticoid receptor occurring after ligand binding and subsequent detachment of the hsp90 from the receptor proper. This model may also be applicable to nuclear receptors. However,a number of major questions still remain ; does hsp90 interact with the glucocorticoid receptor *in vivo*? does hsp90 serve as a transport or storage protein for steroid receptors *in vivo*? and is hsp90 involved as a regulator of steroid hormone receptor activation? To our knowledge, the evidence for the in vivo role of hsp90 have so far been rather indirect. Specific antibodies to steroid receptors and hsp90s as well as their cDNAs which are now rapidly becoming available, will be essential tools to answer these questions, and also to better understand the function of steroid receptors.

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Figure 1

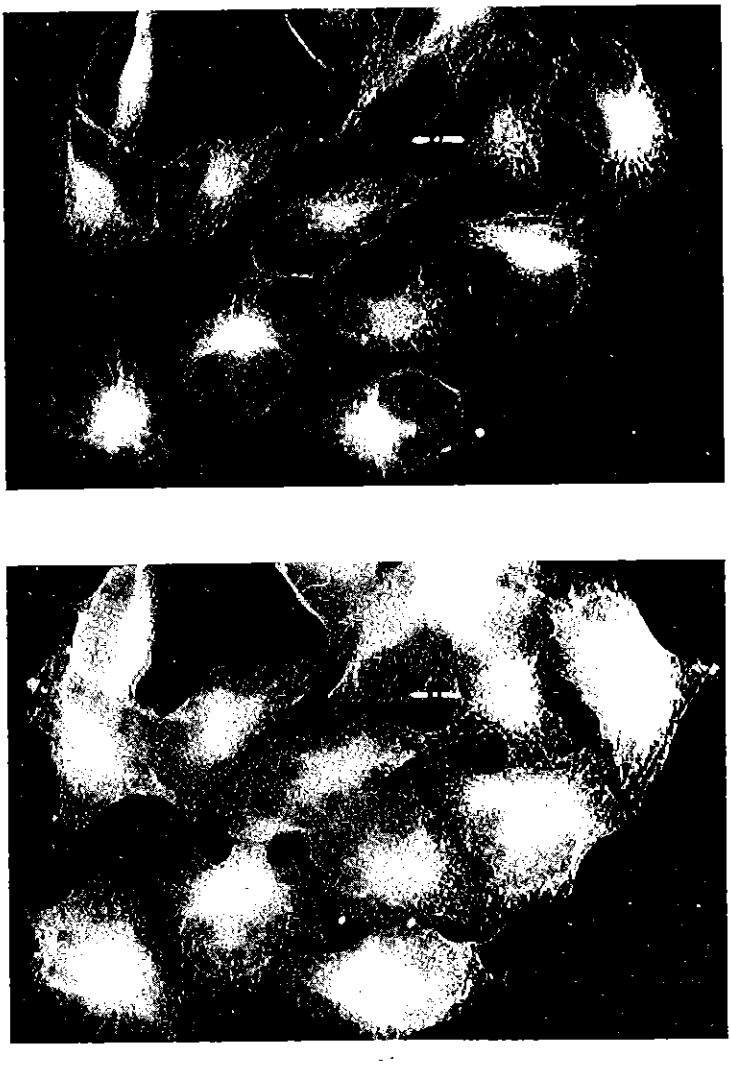

Figure 1

Double immunofluorescence staining of GR and tubulin in NHIK 3025 cells. The cells were fixed with 2 % buffered paraformaldehyde and permeabilized with 0.05 % (v/v) Triton X-100. a) staining visualizes the distribution of tubulin obtained with a rat anti-tubulin antibody. b) The same specimen as in 1a, but using another filter block to detect Texas Red (TR) labeled sheep anti mouse immunoglobulin that has bound to anti GR mab no 7. Note the well

developed cytoplasmic staning in both a and b, as well as the fibrillar pattern of the tubulin staining. There is no overlap of the fluorescence from TR and FITC, respectively.

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