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**6. Phosphorylation of Steroid Receptors**

# GLUCOCORTICOID RECEPTOR PHOSPHORYLATION IN MOUSE L-CELLS

EDWIN R. SANCHEZ*, WILAI TIENRUNGROJ, FRIEDRICH C. DALMAN and ALEXANDER L.-Y. LIN

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

**Summary**—This paper summarizes our observations on the phosphorylation state of untransformed and transformed glucocorticoid receptors isolated from $^{32}$P-labeled L-cells. The 300–350-kDa 9S untransformed murine glucocorticoid receptor complex is composed of a 100-kDa steroid-binding phosphoprotein and one or possibly two units of the 90-kDa heat shock protein (hsp90), which is also a phosphoprotein. Transformation of this complex to the 4S DNA-binding state is accompanied by dissociation of hsp90. When receptors in cytosol are transformed by heating at 25°C, there is no gross change in the degree of phosphorylation of the steroid-binding protein. Both receptors that are bound to DNA after transformation under cell-free conditions and receptors that are located in the nucleus of cells incubated at 37°C in the presence of glucocorticoid are labeled with $^{32}$P. The results of experiments in which the $^{32}$P-labeled receptor was submitted to limited proteolysis suggest that the 16-kDa DNA-binding domain is phosphorylated and that the 28-kDa steroid-binding domain is not.

## INTRODUCTION

A variety of observations made in both intact cells and cell-free lysates indicate that glucocorticoid receptor function may be modulated by phosphorylation–dephosphorylation mechanisms. The first suggestion of this kind was made by Munck *et al.* [1] in 1972. They demonstrated that changes in the steroid-binding capacity of glucocorticoid receptors in intact rat thymocytes correlated with changes in the cellular ATP content. It was proposed that these receptors exist in two functional states and that energy in the form of ATP was required to convert a nonbinding form of the receptor to the steroid-binding state. Similar observations have been made in mouse fibroblasts [2], chick embryo retina cells [3] and human lymphocytes [4]. In addition, it has been shown that the steroid-binding activity of glucocorticoid receptors in cytosol preparations can be inactivated by incubation with purified calf intestine alkaline phosphatase [5, 6]. Phosphatase inhibitors, such as glucose-1-phosphate and fluoride, are able to inhibit (but do not prevent) the temperature-mediated inactivation of steroid receptor binding capacity that is caused by endogenous receptor-inactivating enzymes [7–10].

Although there is indirect evidence that phosphorylation mechanisms play a role in steroid receptor function, there is as yet no direct proof of such regulation. It is clear, however, that a variety of steroid receptors exist as phosphoproteins in the intact cell. This has been shown for glucocorticoid receptors in mouse fibroblasts [11, 12] and rat liver [13, 14] and for progesterone receptors in rabbit uterus [15] and chick oviduct [16, 17]. In the case of the latter, it has been shown that endogenous phosphorylation occurs on serine residues. Although steroid receptors are phosphoproteins, it may be difficult to demonstrate the role of phosphorylation in receptor function, since untransformed steroid receptors exist as large oligomeric complexes composed of at least two distinct phosphoproteins. The untransformed glucocorticoid receptor in mouse L-cells is a heteromer which contains a 100-kDa steroid-binding phosphoprotein and a 90-kDa non-steroid-binding phosphoprotein [12]. The latter protein has been identified as the mammalian 90-kDa heat shock protein [18]. Similarly, the progesterone receptor purified from chick oviduct by affinity chromatography contains a 90-kDa phosphoprotein that does not bind progestins [17, 19]. An antibody raised against this 90-kDa subunit can change the sedimentation velocity of progesterone, glucocorticoid, estrogen and androgen receptor complexes [20, 21]. This 90-kDa progesterone receptor-associated protein has the same peptide map as the chicken 90-kDa heat shock protein (hsp90) and is recognized by an antibody made against hsp90 [22, 23]. Thus, the accumulating evidence points to a general model of steroid receptor structure in which unique steroid-binding phosphoproteins are complexed to a common phosphoprotein which plays a role in the cellular response to stress. Any investigation of the biological significance of phosphorylation states of the steroid binding proteins must closely examine the phosphorylated state of hsp90 as well.

In this paper, we will review our work on phosphorylation of the untransformed and transformed states of the glucocorticoid receptor in cytosol

*To whom correspondence should be addressed.

prepared from mouse L-cells cultured in the presence of [$^{32}$P]orthophosphate. In particular, we will show that the untransformed receptor complex contains a 100-kDa steroid binding phosphoprotein and a 90-kDa phosphoprotein that is immunologically identical to the 90-kDa heat shock protein. Temperature mediated transformation of the complex to generate the DNA-binding state of the receptor results in dissociation of the steroid binding protein from hsp90, and transformation is not accompanied by dephosphorylation of the receptor. When [$^{32}$P]labeled receptors are submitted to limited proteolytic cleavage, the DNA-binding domain contains $^{32}$P but the ligand binding domain ("mero receptor") does not.

### PURIFICATION OF THE $^{32}$P-LABELLED GLUCOCORTICOID RECEPTOR BY IMMUNOADSORPTION TO PROTEIN-A-SEPHAROSE

Our initial attempts to purify the glucocorticoid receptor from cytosol prepared from $^{32}$P-labeled L-cells employed the use of affinity chromatography utilizing deoxycorticosterone–agarose. This approach resulted in the stereospecific elution of two phosphoproteins [11]. The major phosphorylated species migrated at 90 kDa on SDS–PAGE, while the other had a mass of 100 kDa. In order to corroborate this observation, we purified $^{32}$P-labeled receptors by immunoadsorption with the GR49 monoclonal antibody against the glucocorticoid receptor [12]. A typical experiment of this kind is shown in Fig. 1 [24]. In this case, cytosol-containing receptors labeled with [$^{3}$H]dexamethasone mesylate [25] or cytosol prepared from $^{32}$P-labeled cells was incubated with a rabbit antiserum against the mouse glucocorticoid receptor [26], and the antibody–antigen complexes were purified by immunoadsorption to protein-A–Sepharose. It is clear from Fig. 1 that the major form of the L-cell receptor has a mass of approximately 100 kDa. Immunoabsorption of cytosol prepared from $^{32}$P-labeled cells (lane 1) results in the immunospecific presence of two phosphoproteins, one at 100 kDa, and one at 90 kDa. There is no steroid-binding protein migrating at 90 kDa, and in other experiments we demonstrated that the 90-kDa protein does not react with the GR49 monoclonal antibody on immunoblot [12]. This strongly suggested that the 90-kDa protein is different from the steroid-binding protein. In order to determine if the glucocorticoid receptor-associated 90-kDa protein was the same as the progesterone receptor-associated protein, we obtained an antibody against the latter that was prepared in the laboratory of David Toft.

### THE RECEPTOR-ASSOCIATED 90-kDa PHOSPHOPROTEIN IS A MURINE HEAT SHOCK PROTEIN

The monoclonal antibody sent to us by David Toft was designated AC88 (the AC88 antibody was called AC6 in some previous reports from this laboratory) and it was made against an 88-kDa phosphoprotein purified from *Achlya ambisexualis*, a water mold that has steroid receptors which exist as large oligomeric complexes containing a steroid-binding protein in association with one or more moieties of the 88-kDa protein [27]. The AC88 antibody recognizes a 90-kDa phosphoprotein that is part of the untransformed chick oviduct progesterone receptor, as well as 90-kDa proteins present in a variety of human, avian, and rodent cell lines [28]. The experiment of Fig. 2 was performed to determine if the 90-kDa protein associated with the glucocorticoid receptor was recognized by the AC88 monoclonal antibody. In this experiment, L-cell cytosol was incubated with the AC88 antibody or with rabbit anti-receptor serum. After immunoadsorption, the purified proteins were resolved by Western blotting, utilizing as probe antibodies both the AC88 antibody against 90-kDa protein and GR49 monoclonal antibody against the glucocorticoid binding protein (the GR49 antibody was kindly provided by Hannes Westphal). The results show that L-cell cytosol contains a 90-kDa protein that is recognized by AC88 (lane 3) and that this protein is part of the immune-specifically absorbed receptor complex (compare lane 1, pre-immune serum, with lane 2, anti-receptor serum). These observations are in agreement with those recently reported by Mendel *et al.*[29] for the glucocorticoid receptor complex of mouse thymoma cells.

The 90-kDa protein that is immunoadsorbed by AC88 is present in large amounts in cytosols prepared from a wide variety of cell types [18, 28], and it is phosphorylated on serine residues [11]. As the AC88 antibody, which was prepared against a fungal protein, reacts with 90-kDa phosphoproteins in cells from a variety of eukaryotes, including humans, it is clear that elements of the protein have been highly conserved during evolution.

These features are remarkably like those of the mammalian 90-kDa heat shock protein [30]. Accordingly, we obtained from Milton Schlesinger a rabbit antiserum raised against the chicken 89-kDa heat shock protein. This antiserum reacts with proteins of similar size present in human, rat, frog, and *Drosophila* cells [31], and we have used it to demonstrate that the 90-kDa protein associated with the immunoadsorbed glucocorticoid receptor is immunologically identical to the 90-kDa heat shock protein [18]. The polyclonal anti-hsp90 antibody recognizes the same 90-kDa protein that is immunoabsorbed by the AC88 monoclonal antibody [18].

### DISSOCIATION OF THE 90-kDa PHOSPHOPROTEIN FROM THE RECEPTOR OCCURS DURING TRANSFORMATION

Transformation of the glucocorticoid receptor to the DNA-binding state under cell-free conditions is

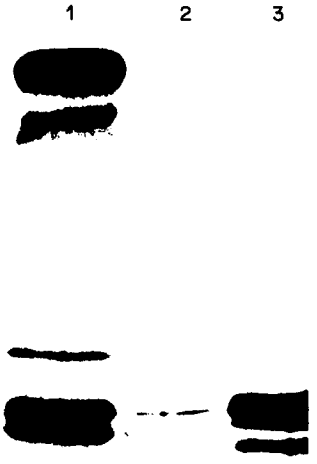


Fig. 1. Comparison of [$^3$H]dexamethasone mesylate-labeled receptor with immunoadsorbed cytosol proteins from $^{32}$P-labeled L-cells. Aliquots (300 $\mu$l) of cytosol incubated with [$^3$H]dexamethasone mesylate or cytosol prepared from $^{32}$P-labeled L-cells were mixed with an equal volume of TES buffer containing 10 mM sodium molybdate and 10 mM dithiothreitol, incubated for 16 h in ice with rabbit anti-receptor antiserum at 5% of final volume, and adsorbed to protein-A–Sephrase. The protein-A–Sepharose pellets were washed sequentially with TES buffer containing 10 mM sodium molybdate and TES buffer containing 400 mM NaCl, 02% Triton X-100, and molybdate. Proteins were extracted from all pellets by boiling in SDS sample buffer, electrophoresed in a 7% polyacrylamide gel, and visualized by autoradiography. Lane 1, immunoadsorbed material from cytosol prepared from L-cells that were incubated with [$^{32}$P]orthophosphate; lane 2, cytosol incubated with [$^3$H]dexamethasone mesylate in the presence of a 100-fold excess of competing nonradioactive dexamethasone; lane 3, cytosol incubated with [$^3$H]dexamethasone mesylate alone. From Sanchez and Pratt[24].

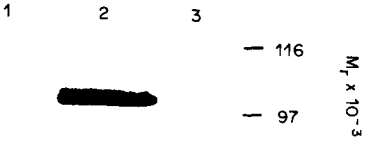


Fig. 2. Immunoblot of L-cell cytosol proteins immunoadsorbed to prtein-A–Sepharose with either rabbit antireceptor antiserum or with the AC88 monoclonal antibody. Aliquots of L-cell cytosol were mixed with equal volumes of TEG buffer containing 10 mM sodium molybdate and 10 mM DTT and incubated for 2 h at 4°C with either the AC88 monoclonal antibody or rabbit sera at the concentrations indicated below. The immune complexes were bound to protein-A–Sepharose and the pellets were washed with TEG buffer containing molybdate and DTT. Samples were electrophoresed in a 7% SDS–polyacrylamide gel and transferred onto nitrocellulose paper. The nitrocellulose paper was washed in blocking buffer, incubated with the AC88 monoclonal antibody at a final concentration of 40 $\mu$g/ml for 11 h at 4°C, and developed by the horseradish peroxidase method. The AC88 monoclonal antibody is specific for the 90-kDa non-steroid-binding phosphoprotein. This process was then repeated on the same nitrocellulose strip, but this time using a 1 : 4 dilution of GR49 hybridoma fluid as the probe antibody. This fluid contains monoclonal antibody specific to the 100-kDa steroid-binding phosphoprotein. Lane 1, 200 $\mu$l of L-cell cytosol incubated with 10% rabbit pre-immune serum. Lane 2, 200 $\mu$l of L-cell cytosol incubated with 10% rabbit antiserum against the mouse L-cell glucocorticoid receptor. Lane 3, 100 $\mu$l of L-cell cytosol incubated with 2% AC88 monoclonal antibody. From Tienrungroj [26].


Fig. 3. Separation of the 90-kDa heat shock phosphoprotein from the 100-kDa steroid-binding phosphoprotein by washing with salt or by temperature-mediated transformation. Cytosol prepared from $^{32}$P-labeled cells was incubated at 0°C with triamcinolone acetonide to prepare steroid-bound receptor and divided into 420-$\mu$l aliquots. One aliquot (lane 4) was incubated for 1 h at 25°C to transform the receptors. Each aliquot was mixed with an equal volume of TEG buffer and incubated with either the BUGR monoclonal antibody against the receptor (lanes 1–4) or the AC88 monoclonal antibody against the 90-kDa heat shock protein (line 5). After adsorption to protein-A–Sepharose, the pellets were washed 7 times with the TEG buffer containing the additions indicated below. Following washing, proteins were extracted by boiling in SDS sample buffer and resolved by SDS–polyacrylamide gel electrophoresis and autoradiography. Lane 1, untransformed receptor immunoadsorbed with BUGR antibody and washed with TEG buffer alone. Lane 2, untransformed receptor adsorbed with BUGR and washed with TEG buffer containing 400 mM NaCl and 0.5% Triton X-100. Lane 3, untransformed receptor adsorbed with BUGR and washed with buffer containing NaCl, Triton X-100 and 10 mM sodium molybdate. Lane 4, transformed receptor adsorbed with BUGR and washed with NaCl, Triton and sodium molybdate. Lane 5, the 90-kDa heat shock protein was immunoadsorbed with AC88 antibody and washed with TEG buffer alone. From Tienrungroj [26].


Fig. 4. Temperature-transformed, DNA-bound glucocorticoid receptor is phosphorylated. An aliquot (1 ml) of $^{32}$P-labeled L-cell cytosol was bound with 50 nM nonradioactive tiamincinolone acetonide for 3 h on ice. The cytosol was then incubated at 25°C for 1 h in order to generate the DNA-binding state of the receptor. Following transformation, the cytosol was diluted with an equal volume of a 12.5% suspension of DNA-cellulose and stirred for 45 min on ice. The DNA-cellulose was washed 3 times with 10 mM Hepes (pH 7.4) buffer, followed by elution of the DNA-bound material with 450 mM NaCl. The salt eluate was diluted with an equal volume of TEG buffer, immunoadsorbed with the BUGR monoclonal antibody, and the protein-A-Sepharose pellet was washed 7 times with TEG buffer containing 400 mM NaCl and 0.5% Triton-X100 followed by 2 washes with TEG buffer alone. The receptor was resolved by electrophoresis on a 7% SDS–polyacrylamide gel and Western blotting. Lane 1 shows the Western blot using the BUGR antibody as the probe and lane 2 shows an autoradiogram made directly from the immunoblot.

accompanied by a reduction in molecular size from approximately 320- kDa to about 100 kDa [32–34]. This has led to the proposal that transformation involves the dissociation of the receptor, either from itself or from other non-receptor subunits. As hsp90 appears to be part of the untransformed receptor complex in cytosol preparations, we have examined the relationship between hsp90 and the steroid-binding protein in the transformation process.

We originally found hsp90 in association with the 100-kDa steroid-binding protein because we were using affinity chromatography to purify the untransformed, non-DNA-binding form of the receptor. Sodium molybdate was included in all of our buffers to preserve the untransformed state of the receptor. We have previously reported that sodium molybdate must be in the buffers used to wash the immunoabsorbed untransformed receptor complex in order to maintain the receptor-hsp90 association [18, 35]. Molybdate is a transition metal oxyanion that can stabilize the steroid-binding form of the glucocorticoid receptor and prevent its transformation to the DNA-binding state [6–9, 36]. As shown in Fig. 3, the association between the 100-kDa steroid-binding protein and hsp90 can be demonstrated in immune complexes absorbed to protein A-Sepharose, if the protein-A–Sepharose pellet is not washed by our usual procedure which includes washing with solutions containing salt and Triton X-100. In lane 1 receptors were adsorbed with the BUGR monoclonal antibody [37] against the receptor (kindly provided by Robert Harrison) and the immunoadsorbed receptors were washed with a low salt buffer. Both the 100- and 90-kDa proteins are present in lane 1. If the immunoadsorbed pellet is washed with salt, the 90-kDa protein is no longer present (lane 2), and if molybdate is present during the salt washes (lane 3), the salt-mediated dissociation of hsp90 is inhibited. As shown in lane 4, hsp90 is not associated with receptors that have been transformed by heating prior to immunoadsorption.

On transformation, the receptor is converted from a 9S non-DNA-binding form to a 4S DNA-binding form. When L-cell cytosol is heated at 25°C, the reduction in size of the receptor and acquisition of DNA-binding activity occur only if the receptor is bound by steroid. We have also shown that temperature-mediated dissociation of hsp90 from the receptor is a ligand-dependent event [38]. In contrast to temperature-mediated transformation, salt causes both dissociation observations are consistent with the proposal that dissociation of hsp90 from the receptor occurs during transformation of the receptor to the DNA-binding form.

It is likely that dissociation of the receptor from hsp90 accounts for the change in charge of the receptor that occurs with transformation to the DNA-binding state [39]. Untransformed receptors are more negatively charged than transformed receptors and molybdate stabilizes this negatively charged form as determined by elution from DEAE [40]. The 90-kDa heat shock protein is also negatively charged and it elutes from DEAE columns at exactly the salt concentration required for elution of the untransformed receptor [38].

## THE DNA-BINDING FORM OF THE GLUCOCORTICOID RECEPTOR IS PHOSPHORYLATED

Several laboratories have suggested that temperature-mediated transformation of steroid receptors to generate the DNA-binding state may require receptor dephosphorylation [41–43]. These suggestions were based on indirect evidence derived from studies in both intact cells and cell-free lysates. More recently, direct but conflicting reports on the phosphorylation states of transformed progesterone receptors have been published. Logeat *et al.*[15], working with receptors located in cytosolic and nuclear fractions from rabbit uterine slices incubated with [$^{32}$P]orthophosphate concluded that the receptor isolated from the nucleus had undergone an increase in phosphorylation that was steroid dependent. However, Garcia *et al.*[44], have reported that the progesterone receptor present in the nucleus of chick oviduct cells cultured in the presence of [$^{32}$P]orthophosphate was not phosphorylated. In contrast, receptor isolated from the cytoplasm of the same cells did contain radiolabeled phosphate.

In order to determine if phosphorylation plays a role in the DNA-binding function of glucocorticoid receptors, we have examined the phosphorylation state of L-cell receptors that have been transformed and bound to DNA under cell-free conditions. We have also examined receptors extracted from the nuclei of intact cells exposed to glucocorticoid at 37°C. An experiment carried out under cell-free conditions is shown in Fig. 4. In this experiment, cytosol made from L-cells grown in the presence of [$^{32}$P]orthophosphate was bound with non-radioactive steroid and transformed to the DNA-binding state by incubation at 25°C for 1 h. After incubation with DNA-cellulose and washing of the DNA-cellulose pellet, the DNA-bound proteins were eluted with salt. The salt-extracted receptor was isolated by immunoabsorption with the BUGR monoclonal antibody, and the DNA-binding form of the receptor was resolved by electrophoresis of a denaturing gel, followed by Western blotting and autoradiography. The results clearly demonstrate the presence of receptor protein (lane 1) and the presence of radiolabeled phosphate in the receptor band (lane 2).

We have salt-extracted receptors from nuclei prepared from $^{32}$P-labeled cells. After immunoadsorption and resolution by electrophoresis on denaturing gels, $^{32}$P-labeled receptors can be readily detected in the nuclear salt extract [26]. The techniques employed in these experiments are only semi-quantitative, and we cannot at this time

determine if the nuclear-bound receptor had undergone additional phosphorylation with respect to the cytosolic form, as suggested by Logeat *et al.*[15]. It is clear, however, that the DNA-binding form of the L-cell glucocorticoid receptor is phosphorylated. Whether the phosphorylation state can actually affect the DNA-binding capacity of the receptor requires further experimentation. It should be noted that Auricchio *et al.*[45, 46] have identified the presence of a phosphatase in uterine cell nuclei that dephosphorylates the estrogen receptor and the existence of a uterine nuclear kinase that phosphorylates the progesterone receptor is suggested by the observations of Logeat *et al.*[15].

### LIMITED PROTEOLYSIS OF THE $^{32}$P-LABELED RECEPTOR

Several laboratories have used limited proteolysis to characterize the ligand-binding and DNA-binding domains of the glucocorticoid receptor [47–51]. Digestion with chymotrypsin produces a fragment with a mass of about 42 kDa that contains the steroid-binding site and binds to DNA. Digestion with trypsin yields both a 28-kDa steroid-bound fragment that has lost the ability to bind to DNA and a 16-kDa fragment that does not contain bound steroid but reacts with the BUGR monoclonal antibody and binds to DNA [52]. With immunological techniques, it is possible to detect the existence of a third domain that is separate from the steroid-binding and DNA-binding domains but contains epitopes that are recognized by a number of anti-receptor antibodies [53].

We are digesting receptors in cytosol prepared from $^{32}$P-labeled L-cells in order to determine which of these domains are phosphorylated. These studies are at an early stage of development, but our initial observations suggest that the 28-kDa steroid-binding domain is not phosphorylated and that the 16-kDa DNA-binding domain is phosphorylated. In the experiment of Fig. 5, $^{32}$P-labeled receptors in L-cell cytosol were incubated with the BUGR monoclonal antibody and adsorbed to protein-A–Sepharose. The immunoadsorbed protein-A–Sepharose pellet was then incubated on ice with buffer (lane 1), trypsin (lane 2), or with chymotrypsin (lane 3). The prominent chymotryptic fragment at 42-kDa is seen in the immunoblot in lane 3 of panel A and the $^{32}$P radioactivity at the same location is seen in the autoradiogram in panel B of the figure. The 16-kDa tryptic fragment is indicated by the arrow in lane 2 of both the immunoblot and the autoradiogram. Although we can readily demonstrate the 28-kDa trypsin fragment by cleaving the [$^3$H]dexamethasone mesylate-bound receptor (not shown), we have not seen $^{32}$P-labeling of that fragment.

It is difficult to obtain the 16-kDa fragment in good yield by digesting immunoadsorbed receptors with trypsin. This 16-kDa fragment is obtained in higher yield, however, if receptors in cytosol are first digested with trypsin and the fragment is subsequently immunoadsorbed with the BUGR antibody as shown in Fig. 6. In this experiment, the 16-kDa trypsin fragment was incubated with calf intestine alkaline phosphatase at 0°C after immunoadsorption. It is clear from lane 1 of panel B that the 16-kDa tryptic fragment is radiolabeled, and the fact that the intensity of the label is reduced by incubation with alkaline phosphatase (lane 2) suggests that the $^{32}$P is covalently bound.

Taken together, our observations after limited proteolysis of the receptor suggest that the 16-kDa DNA-binding domain is phosphorylated and that the 28-kDa steroid-binding domain is not. We do not yet know how many sites on the receptor are phosphorylated and we do not know if there are phosphorylated sites in the third domain that is separate from the steroid-binding and DNA-binding domains.

### A MODEL OF RECEPTOR TRANSFORMATION AS IT OCCURS IN CYTOSOL PREPARATIONS

The mechanism of transformation of the glucocorticoid receptor complex to the DNA-binding state is summarized in the model presented in Fig. 7. It can be seen that the untransformed receptor is a complex of 300–350-kDa that contains two phosphoproteins. One phosphoprotein is the 100-kDa steroid-binding protein, the other subunit is the non-steroid-binding murine heat shock protein (hsp90) [12, 18]. The bound steroid is indicated by the hexagon. The open circles with question marks are meant to indicate that other, as yet undefined, components of the untransformed complex may exist. During transformation, the 100-kDa steroid-binding unit dissociates from hsp90, resulting both in reduction in the size of the complex and the exposure of positive charges on the surface of the receptor protein that react with DNA and other polyanions [29, 38]. Transformation of the receptor to the DNA-binding state can be measured in terms of the dissociation of hsp90 from the steroid-binding subunit [38].

We have not observed any dephosphorylation of the 100-kDa glucocorticoid-binding protein during transformation, either in the intact cell or under cell-free conditions [26]. It should be mentioned, however, that our techniques would detect only rather large changes in phosphorylation. As the DNA-bound steroid–receptor complex that is formed under cell-free conditions also contains phosphate, it is clear that the receptor does not have to be in a dephosphorylated state in order to bind to DNA.

The presence of SH groups in the model is to illustrate that sulfhydryl moieties are absolutely required for the glucocorticoid receptor to bind steroid [43, 54] and for the transformed receptor to

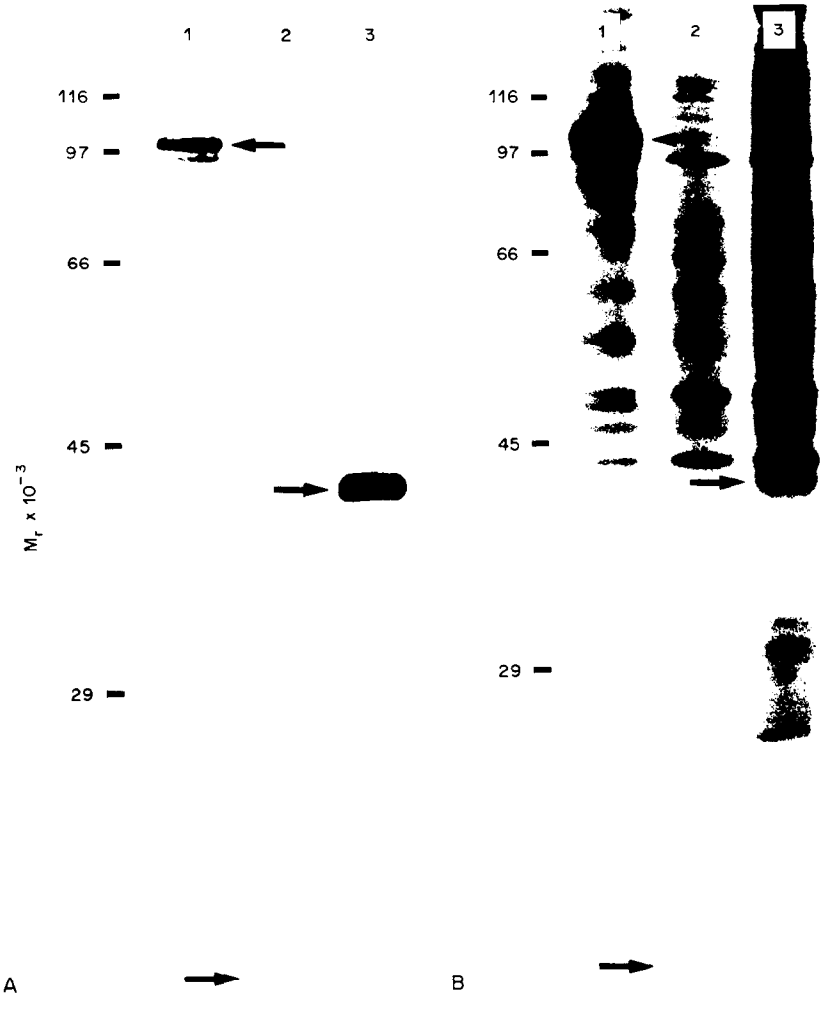


Fig. 5. Proteolytic cleavage of the $^{32}$P-labeled glucocorticoid receptor. Aliquots (500 $\mu$l) of $^{32}$P-labeled L-cell cytosol were diluted 1 : 1 with TEG buffer and made 2% for the BUGR anti-receptor monoclonal antibody. The antibody was bound for 3 h on ice, followed by immunoadsorption to protein-A–Sepharose. After extensive washing, the immunoadsorbed proteins were either eluted with SDS sample buffer or digested with trypsin or chymotrypsin at 0°C for 1 h at the concentrations indicated below. Proteolysis was stopped by the addition of SDS sample buffer and boiling. All samples were subjected to SDS–polyacrylamide gel electrophoresis followed by Western transfer to nitrocellulose paper and immunoblotting. The BUGR antibody was used as a probe. (A) Western blot of the protease-digested receptor: lane 1, undigested; lane 2, digestion with 20 $\mu$g/ml trypsin; lane 3, 10 $\mu$g/ml chymotrypsin. The arrows indicate the immunoreactive receptor protein band obtained for each condition. (B) autoradiogram made directly from the Western blot seen in (A). The arrows indicate those $^{32}$P-labeled bands that are superimposable on the receptor proteins in the immunoblot.

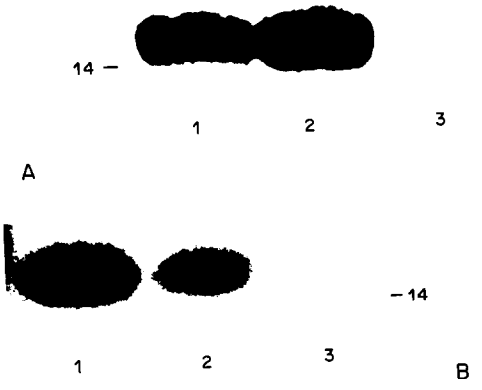


Fig. 6. Trypsin digestion of the $^{32}$P-labeled glucocorticoid receptor in whole cytosol to generate the 16-kDa DNA-binding fragment. Aliquots (500 $\mu$l) of L-cell cytosol were digested with 100 $\mu$g/ml trypsin at 0°C for 1 h. Digestion was stopped by the addition of soybean trypsin inhibitor. The aliquots were diluted with an equal volume TEG buffer and made 2% for either BUGR antibody or nonspecific mouse IgG. The samples were then immunoadsorbed, and analyzed by Western blotting and autoradiography as described in the legend to Fig. 5. (A) Western blot of the receptor digested with trypsin; lane 1, trypsin digest immunoadsorbed with BUGR antibody; lane 2, trypsin digest immunoadsorbed with BUGR followed by incubation of the immunoadosrbed pellet with calf intestine alkaline phosphatase (100 $\mu$g/ml) for 1 h at 0°C; lane 3, trypsin digest immunoadsorbed with mouse IgG. (B) Autoradiogram made directly from the Western blot shown in (A).

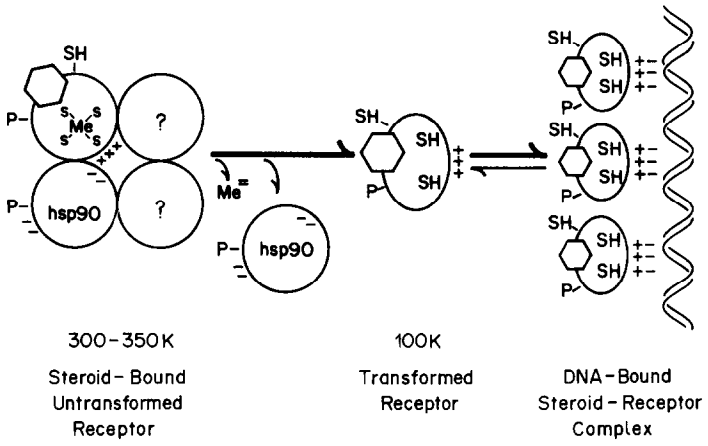


Fig. 7. Model illustrating the components of the untransformed and transformed glucocorticoid receptor and the dissociation of the subunits during transformation. The receptor-bound steroid is indicated by the hexagon on the 100-kDa phosphoprotein containing the potential metal-binding site. The 90-kDa non-steroid-binding subunit has been identified as the murine 90-kDa heat-shock protein (hsp 90). The rationale for this model is presented in the text.

bind to DNA [55]. The sulfhydryl moieties that are required for steroid binding are different from the sulfhydryl moieties required for DNA binding [56]. The requirement for different sulfhydryl groups for each function is also indicated in the model.

Both molybdate and an endogenous, heat-stable, cytosolic factor prevent temperature-mediated transformation to the DNA-binding state and subunit dissociation [38]. There is good evidence that the endogenous factor is a metal or a metal containing-complex (Moshinchi, Sanchez, Grippo and Pratt, in preparation), and it is possible that the factor and molybdate occupy the same metal-binding site in the receptor (Me-S). The mechanism by which molybdate and the endogenous factor inhibit transformation is unclear. Interestingly, potential metal-binding domains have been identified in several DNA-binding proteins [57]. These domains contain cysteine residues which provide sulfur moieties through which a tetrahedral metal coordination complex could be formed. Similar potential metal-binding domains have now been identified in the primary structure of the DNA-binding domains of glucocorticoid [58] and estrogen [59] receptors.

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## REFERENCES

1. Munck A., Wira C., Young D. A., Mosher K. M., Hallahan C. and Bell P. A.: Glucocorticoid-receptor complexes and the earliest steps in the action of glucocorticoids on thymus cells. *J. steroid Biochem.* **3** (1972) 567–578.
2. Ishii D. N., Pratt W. B. and Aronow L.: *In vitro* degradation and stabilization of the glucocorticoid binding component from mouse fibroblasts. *Biochemistry* **11** (1972) 3896–3904.
3. Chader G. J.: Some factors affecting the uptake, binding and retention of [$H^3$]cortisol by the chick embryo retina as related to enzyme induction. *J. Neurochem.* **21** (1973) 1525–1532.
4. Wheeler R. H., Leach K. L., LaForest A. C., O'Toole T. E., Wagner R. and Pratt W. B.: Glucocorticoid receptor activation and inactivation in cultured human lymphocytes. *J. biol. Chem.* **256** (1981) 434–441.
5. Nielsen C. J., Sando J. J. and Pratt W. B.: Evidence that dephosphorylation inactivates glucocorticoid receptors. *Proc. natn. Acad. Sci. U.S.A.* **74** (1977) 1398–1402.
6. Housley P. R., Dahmer M. K. and Pratt W. B.: Inactivation of glucocorticoid binding capacity by protein phosphatases in the presence of molybdate and complete reactivation by dithiothreitol. *J. biol. Chem.* **257** (1982) 8615–8618.
7. Nielsen C. J., Sando J. J., Vogel W. M. and Pratt W. B.: Glucocorticoid receptor inactivation under cell-free conditions. *J. biol. Chem.* **252** (1977) 7568–7578.
8. Leach K. L., Dahmer M. K., Hammond N. D., Sando, J. J. and Pratt W. B.: Molybdate inhibition of glucocorticoid receptor inactivation and transformation. *J. biol. Chem.* **254** (1979) 11,884–11,890.
9. Toft D. and Nishigori H.: Stabilization of the avian progesterone receptor by inhibitors. *J. steroid Biochem.* **11** (1979) 413–416.
10. Auricchio F. and Migliaccio A.: *In vitro* inactivation of oestrogen receptor by nuclei. Prevention by phosphatase inhibitors. *FEBS Lett.* **117** (1980) 224–226.
11. Housley P. R. and Pratt W. B.: Direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. *J. biol. Chem.* **258** (1983) 4630–4635.
12. Housley P. R., Sanchez E. R., Westphal H. M., Beato M. and Pratt W. B.: The molybdate-stabilized L-cell glucocorticoid receptor isolated by affinity chromatography or with a monoclonal antibody is associated with a 90–92-kDa non-steroid-binding phosphoprotein. *J. biol. Chem.* **260** (1985) 13,810–13,817.
13. Grandics P., Miller A., Schmidt T. J. and Litwack G.: Phosphorylation *in vivo* of rat hepatic glucocorticoid receptor. *Biochem. biophys. Res. Commun.* **120** (1984) 59–65.
14. Singh V. B. and Mondgil V. K.: Phosphorylation of the rat liver glucocorticoid receptor. *J. biol. Chem.* **260** (1985) 3684–3690.
15. Logeat F., Le Cunff M., Pamphile R. and Milgrom E.:

The nuclear-bound form of the progesterone receptor is generated through a hormone-dependent phosphorylation. *Biochem. biophys. Res. Commun.* **131** (1985) 421–427.
16. Dougherty J. J., Puri R. K. and Toft D. O.: Phosphorylation *in vivo* of chicken oviduct progesterone receptor. *J. biol. Chem.* **257** (1982) 14,226–14,230.
17. Dougherty J. J., Puri R. K. and Toft D. O.: Polypeptide components of two 8S forms of chicken oviduct progesterone receptor. *J. biol. Chem.* **259** (1984) 8004–8009.
18. Sanchez E. R., Toft D. O., Schlesinger M. J. and Pratt W. B.: The 90-kDa non-steroid-binding phosphoprotein that binds to the untransformed glucocorticoid receptor in molybdate-stabilized L-cell cytosol is the murine 90-kDa heat shock protein. *J. biol. Chem.* **260** (1985) 12,398–12,401.
19. Renoir J. M., Buchou T., Mester J., Radanyi C. and Bauleiu E. E.: Oligomeric structure of molybdate-stabilized, nontransformed 8S progesterone receptor from chick oviduct cytosol. *Biochemistry* **23** (1984) 6016–6023.
20. Joab I., Radanyi C., Renoir M., Buchou T., Catelli M. G., Binart N., Mester J. and Baulieu E. E.: Common non-hormone-binding component in nontransformed chick oviduct receptors of four steroid hormones. *Nature, Lond.* **308** (1984) 850–853.
21. Sullivan W. P., Vroman B. T., Bauer V. J., Puri R. K., Riehl R. M., Pearson G. R. and Toft D. O.: Isolation of a steroid receptor binding protein from the chicken oviduct and production of monoclonal antibodies. *Biochemistry* **24** (1985) 4214–4222.
22. Schuh S., Yonemoto W., Brugge J., Bauer J. S., Riehl R. M., Sullivan W. P. and Toft D. O.: A 90,000-dalton binding protein common to both steroid receptors and the Ross sarcoma virus transforming protein, $pp60^{v\text{-}src}$. *J. biol. Chem.* **260** (1985) 14,292–14,296.
23. Catelli M. G., Binart N., Jung-Testas I., Renoir J. M., Baulier E. E., Feramisco J. R. and Welch W. J.: The common 90-kDa protein component of non-transformed 8S steroid receptors is a heat-shock protein. *EMBO J.* **4** (1985) 3131–3135.
24. Sanchez E. R. and Pratt W. B.: Phosphorylation of L-cell glucocorticoid receptors in immune complexes: evidence that the receptor is not a protein kinase. *Biochemistry* **25** (1986) 1378–1382.
25. Simmons S. S., Jr. and Thompson E. B.: Dexamethasone 21-mesylate: an affinity label of glucocorticoid receptors from rat hepatoma tissue culture cells. *Proc. natn. Acad. Sci. U.S.A.* **78** (1981) 3541–3545.
26. Tienrungroj W.: *Glucocorticoid Receptor Transformation and DNA Binding*. Ph.D. thesis, University of Michigan (1986).
27. Riehl R. M. and Toft D. O.: Analysis of the steroid receptor of *Achlya ambisexualis*. *J. biol. Chem.* **259** (1984) 4534–4541.
28. Riehl R. M., Sullivan W. P., Vroman B. T., Bauer V. J., Pearson G. R. and Toft D. O.: Immunological evidence that the non-hormone binding component of avian steroid receptors exists in a wide range of tissues and species. *Biochemistry* **24** (1985) 6586–6591.
29. Mendel D. B., Bodwell J. E., Gametchu B., Harrison R. W. and Munck A.: Molybdate-stabilized nonactivated glucocorticoid receptor complexes contain a 90-kDa non-steroid-binding phosphoprotein that is lost on activation. *J. biol. Chem.* **261** (1986) 3758–3763.
30. Craig E.: The heat shock response. *CRC Crit. Rev. Biochem.* **18** (1986) 239–280.
31. Kelley P. M. and Schlesinger M. J.: Antibodies to two major chicken heat shock proteins react with similar proteins in widely divergent species. *Molec. cell. Biol.* **2** (1982) 267–274.
32. Holbrook N. J., Bodwell J. E., Jeffries M. and Munck A.: Characterization of nonactivated and activated glucocorticoid–receptor complexes from intact rat thymus cells. *J. biol. Chem.* **258** (1983) 6477–6485.
33. Sherman M. R., Moran M. C., Tuazon F. B. and Stevens Y. W.: Structure, dissociation, and proteolysis of mammalian steroid receptors. *J. biol. Chem.* **258** (1983) 10,366–10,377.
34. Vedeckis W. V.: Subunit dissociation as a possible mechanism of glucocorticoid receptor activation. *Biochemistry* **22** (1983) 1983–1989.
35. Sanchez E. R., Housley P. R. and Pratt W. B.: The molybdate-stabilized glucocorticoid binding complex of L-cells contains a 98–100 kdalton steroid binding phosphoprotein and a 90 kdalton nonsteroid-binding phosphoprotein that is part of the murine heat-shock complex. *J. steroid Biochem.* **24** (1986) 9–18.
36. Dahmer M. K., Housley P. R. and Pratt W. B.: Effects of molybdate and endogenous inhibitors on steroid receptor inactivation, transformation and translocation. *A. Rev. Physiol.* **46** (1984) 67–81.
37. Gametchu B. and Harrison R. W.: Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. *Endocrinology* **114** (1984) 274–279.
38. Sanchez E. R., Meshinchi S., Tienrungroj W., Schlesinger M. J., Toft D. O. and Pratt W. B.: Relationship of the 90-kDa murine heat shock protein to the physical properties of the untransformed and transformed states of the L-cell glucocorticoid receptor. Submitted.
39. Sakaue Y. and Thompson E. B.: Characterization of two forms of glucocorticoid hormone–receptor complex separated by DEAE-cellulose column chromatography. *Biochem. biophys. Res. Commun.* **77** (1977) 533–541.
40. Dahmer M. K., Quasney M. W., Bissen S. T. and Pratt W. B.: Molybdate permits resolution of untransformed glucocorticoid receptors from the transformed state. *J. biol. Chem.* **256** (1981) 9401–9405.
41. Munck A. and Leung K.: Glucocorticoid receptors and mechanisms of action. In *Receptors and Mechanism of Action of Steroid Hormones* (Edited by J. R. Pasqualini). Marcel Dekker, New York (1977) pp. 311–397.
42. Barnett C. A., Schmidt T. J. and Litwack G.: Effects of calf intestinal alkaline phosphatase, phosphatase inhibitors, and phosphorylated compounds on the rate of activation of glucocorticoid–receptor complexes. *Biochemistry* **19** (1980) 5446–5455.
43. Sando J. J., Hammond N. D., Stratford C. A. and Pratt W. B.: Activation of thymocyte glucocorticoid receptors to the steroid binding form. *J. biol. Chem.* **254** (1979) 4779–4789.
44. Carcia T., Jung-Testas I. and Baulieu E. E.: Lack of phosphorylation of nuclear tightly bound progesterone receptor in primary chick oviduct cultures. *Proc. natn. Acad. Sci. U.S.A.* (1986) in press.
45. Auricchio F., Migliaccio A. and Rotondi A.: Inactivation of estrogen receptor *in vitro* by nuclear dephosphorylation. *Biochem. J.* **194** (1981) 569–574.
46. Auricchio F., Migliaccio A. and Castoria A.: Dephosphorylation of estradiol receptor *in vitro*. A hypothesis on the mechanism of action of non-steroidal anti-oestrogens. *Biochem. J.* **198** (1981) 699–702.
47. Wrange O. and Guftafsson J. A.: Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. *J. biol. Chem.* **253** (1978) 856–865.
48. Gehring U. and Hotz A.: Photoaffinity labeling and partial proteolysis of wild-type and variant glucocorticoid receptors. *Biochemistry* **22** (1983) 4013–4018.
49. Vedeckis W. V.: Limited proteolysis of the mouse liver glucocorticoid receptor. *Biochemistry* **22** (1983) 1975–1983.
50. Reichman M. E., Foster C. M., Eisen L. P., Eisen H. J.,

Torain B. F. and Simons S. S.: Limited proteolysis of covalently labeled glucocorticoid receptors as a probe of receptor structure. *Biochemistry* **23** (1984) 5376–5384.

51. Wrange O., Okret S., Radojcic M., Carlstedt-Duke J. and Gustafsson J. A.: Characterization of the purified activated glucocorticoid receptor from rat liver cytosol. *J. biol. Chem.* **259** (1984) 4534–4541.
52. Eisen L. P., Reichman M. E., Thompson E. B., Gametchu B., Harrison R. W. and Eisen H. J.: Monoclonal antibody to the rat glucocorticoid receptor: relationship between the immune reactive and DNA-binding domain. *J. biol. Chem.* **260** (1985) 11,805–11,810.
53. Carstedt-Duke J., Okret S., Wrange O. and Gustafsson J. A.: Immunochemical analysis of the glucocorticoid receptor: identification of a third domain separate from the steroid-binding and DNA-binding domains. *Proc. natn. Acad. Sci. U.S.A.* **79** (1982) 4260–4264.
54. Grippo J. F., Holmgren A. and Pratt W. B.: Proof that the endogenous, heat-stable glucocorticoid receptor-activating factor is thioredoxin. *J. biol. Chem.* **260** (1985) 93–97.
55. Bodwell J. E., Holbrook N. J. and Munck A.: Sulfhydryl-modifying reagents reversibly inhibit binding of glucocorticoid–receptor complexes to DNA-cellulose. *Biochemistry* **23** (1984) 1392–1398.
56. Bodwell J. E., Holbrook N. J. and Munck A.: Evidence for distinct sulfhydryl groups associated with steroid- and DNA-binding domains of rat thymus glucocorticoid receptors. *Biochemistry* **23** (1984) 4237–4242.
57. Berg J. M.: Potential metal-binding domains in nucleic acid binding proteins. *Science* **232** (1986) 485–487.
58. Hollenberg S. M., Wienberger C., Ong E. S., Cerelli G., Oro A., Lebo R., Thompson E. B., Rosenfeld M. A. and Evans R. M.: Primary structure and expression of a functional human glucocorticoid receptor DNA. *Nature, Lond.* **318** (1985) 635–641.
59. Greene G. L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine J.: Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** (1986) 1150–1154.