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EVIDENCE FROM PULSE-CHASE LABELING STUDIES THAT THE ANTIGLUCOCORTICOID HORMONE RU486 STABILIZES THE NONACTIVATED FORM OF THE GLUCOCORTICOID RECEPTOR IN MOUSE LYMPHOMA CELLS

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Summary—A pulse-chase labeling technique was used to determine the properties of glucocorticoid receptors occupied by the antiglucocorticoid hormone RU486 in S49.1 mouse lymphoma cells. Cells were pulse-labeled with [^{35}S]methionine and then at the beginning of the chase, either no hormone (control), dexamethasone, or RU486 was added to cells. At 4 h into the chase, cytosol was prepared and receptors were immunoadsorbed to protein A-Sepharose using the BuGR2 antireceptor antibody. Immunoadsorbed proteins were resolved by gel electrophoresis and analyzed by autoradiography. The 90 kDa heat shock protein (hsp90) coimmunoadsorbed with receptors from control cells when protein A-Sepharose pellets were washed with 250 mM NaCl but not when protein A-Sepharose pellets were washed with 500 mM NaCl, indicating that hsp90–receptor complexes are disrupted by a high concentration of salt in the absence of molybdate. hsp90 coimmunoadsorbed with receptors from RU486-treated cells even when protein A-Sepharose pellets were washed with 500 mM NaCl, indicating that RU486 stabilizes the association of hsp90 with the glucocorticoid receptor. In contrast, hsp90 did not coimmunoadsorb with receptors from dexamethasone-treated cells, consistent with earlier evidence that hsp90 dissociates from the receptor when the receptor binds glucocorticoid hormone. Dexamethasone induced a rapid quantum decrease in the amount of normal receptor recovered from cytosol but did not induce a decrease in the amount of nuclear transfer deficient receptor recovered from cytosol, consistent with tight nuclear binding of normal receptors occupied by dexamethasone. In contrast, RU486 did not induce a quantum decrease in the recovery of normal receptors from cytosol, indicating that receptors occupied by RU486 are not tightly bound in the nuclear fraction.

We conclude that the antiglucocorticoid hormone RU486, in contrast to the glucocorticoid hormone dexamethasone, stabilizes the association between the glucocorticoid receptor and hsp90. The decreased affinity of receptors occupied by RU486 for the nuclear fraction may be due to their association with hsp90 and may account for the failure of RU486 to exert agonist activity.

INTRODUCTION

The glucocorticosteroid receptor is an important regulatory molecule that interacts with specific enhancer sequences on DNA and thereby regulates gene transcription [1]. The role of the glucocorticoid hormone appears to be that of an effector molecule that promotes conversion of the receptor from a non-DNA-binding form to a DNA-binding form. Certain steroid hormones antagonize the effects of glucocorticoid hormones and are, therefore, referred to as antiglucocorticoids. One such hormone, RU486, binds to the glucocorticoid receptor with high affinity but does not display agonist activity [2].

A number of studies have identified differences between the properties of RU486–receptor complexes and glucocorticoid–receptor complexes that might explain the failure of RU486 to exert agonist activity. Although RU486–receptor complexes bind to both nonspecific and specific DNA sequences *in vitro*, the affinity of RU486–receptor complexes for DNA appears to be less than that of glucocorticoid–receptor complexes [2–7]. Whole cell autoradiographic and immunocytochemical studies indicate

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Abbreviations and trivial names used: PBS, phosphate buffered saline; kDa, kilodalton; dexamethasone, 9α-fluoro-16α-methyl-11β, 17α, 21-trihydroxypregna-1,4-diene-3,20-dione; RU486, 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(prop-1-ynyl)estra-4,9-diene-3-one; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

that RU486–receptor complexes, like glucocorticoid–receptor complexes, localize to the cell nucleus [8, 9]. However, results of subcellular fractionation experiments suggest that the magnitude of nuclear uptake is significantly less for RU486–receptor complexes than for glucocorticoid–receptor complexes [3, 2, 10–12].

More recent studies have focused on the effect of RU486 on the process through which the glucocorticoid receptor is converted from a non-DNA-binding form to a DNA-binding form. This process, which is often referred to as receptor activation or transformation, takes place in the presence of glucocorticoid hormone and is accompanied by dissociation of the receptor from an oligomeric form to a monomeric form [13, 14]. There is considerable evidence that the oligomer is a hetero-oligomer having both a hormone-binding receptor subunit and a non-hormone-binding subunit which has been identified as the 90 kDa heat shock protein, hsp90 [15–19]. RU486 appears to stabilize the association between hsp90 and the receptor, thus impeding the conversion of receptors to a DNA-binding form [11, 12].

To further investigate the mechanism of RU486 action in cells, we have used a pulse-chase labeling method to characterize the intracellular dynamics of the glucocorticoid receptor. Our results provide additional evidence that RU486 stabilizes the association between the glucocorticoid receptor and hsp90 and that RU486–receptor complexes do not bind as tightly in the nucleus as glucocorticoid–receptor complexes.

EXPERIMENTAL PRODEDURES

Chemicals

Acrylamide and other chemicals for gel electrophoresis were from Bio-Rad. The low molecular weight standards for gel electrophoresis, from Pharmacia LKB Biotechnology Inc., were phosphorylase b (97.4 kDa), albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and a-lactalbumin (14.4 kDa). Protein A-Sepharose, Triton X-100, and other chemicals were from Sigma. Tween 20 was from Bio-Rad. [^{35}S]methionine (1192 Ci/mmol) was from Amersham.

Buffers

Phosphate buffered saline (PBS), pH 7.8 (25°C), contained 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 15 mM KH_2PO_4. Buffer A contained 25 mM Tris, pH 8.2 (4°C), 1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol. Buffer B contained 25 mM Tris, pH 8.2 (4°C), 1 mM EDTA, 500 mM NaCl, 10% glycerol, 0.2% Triton X-100. Buffer C was the same as Buffer B except that it contained 250 mM NaCl. The sample buffer for gel electrophoresis contained 0.6 M Tris, pH 8.85 (25°C), 2% sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and bromphenol blue.

Cell culture

The S49.1 mouse lymphoma cell line was obtained from American Type Culture Collection. The nuclear transfer deficient variant cell line, S49.22r, was from Dr Keith Yamamoto, University of California, San Francisco. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, penicillin, and streptomycin. DMEM and serum were purchased from Biologos. Cells were maintained at 37°C in a 5% CO_2, 95% air atmosphere and were used for experiments during the exponential phase of growth. Where stated, serum was extracted with charcoal to remove endogenous cortisol using a previously described method [20].

Antibodies

Crude tissue culture supernatant from the BuGR-2 hybridoma clone was a gift of Dr Robert Harrison, University of Arkansas for Medical Sciences, Little Rock, Ariz. The BuGR-2 clone produces IgG_2 monoclonal antibodies that react with an epitope located in or near the DNA-binding site of mouse and rat glucocorticoid receptors [21–24]. Ascites fluid containing AC88 monoclonal antibody was a gift of Dr David Toft, Mayo Clinic, Rochester, Minn. AC88 has a broad spectrum of species cross-reactivity and recognizes the 90-kDa heat shock protein (hsp90) common to steroid hormone receptors [25].

Pulse-chase labeling

Cells were labeled using a modification of a previously described method [26]. Cells were gently pelleted, resuspended at a concentration of 5 million cells/ml in methionine-free DMEM (Biologos) supplemented with 10% heat-inactivated fetal calf serum, 0.4 mM glutamine, penicillin, streptomycin, and then incubated for 1 h at 37°C in a 5% CO_2, 95% air atmosphere. The cells were then gently pelleted and resuspended at a concentration of 50 million cells/ml in the same methionine-deficient medium. [^{35}S]Methionine (1192 Ci/mmol) was added to give a final concentration of 50 μCi/ml, and the cells were incubated for 30 min at 37°C in a 5% CO_2, 95% air atmosphere. The cells were then gently pelleted and resuspended at a concentration of 8–10 million cells/ml in DMEM supplemented with 1 mg/ml unlabeled methionine plus 10% heat-inactivated fetal calf serum, 4 mM glutamine, penicillin, and streptomycin. The cell suspension was divided into equal aliquots in tissue culture flasks and incubated at 37°C in a 5% CO_2, 95% air atmosphere for a period of time referred to as the chase. At the beginning of the chase and at each time point during the chase, all of the cells were removed from a single flask and cytosol was prepared for the immunoadsorption assay described below.

Steroid hormones

Dexamethasone (Sigma) was dissolved in absolute ethanol to give a stock solution with a concentration of 4.1 mM. RU486 (provided by Dr R. Deraedt at the Centre de Recherches Roussel–Uclaf, Romainville, France) was dissolved in absolute ethanol to give a stock solution with a concentration of 4.1 mM. In experiments involving the addition of one of these hormones to cells, the stock solutions were diluted in sterile culture medium and added to cultures at the zero hour time point in the chase. An equivalent amount of absolute ethanol diluted in culture medium was added to control cultures.

Cytosol preparation

The method of cytosol preparation was modified after that of Mendel *et al.* [17]. Cells were washed twice with PBS at 4°C and after the final wash pelleted by centrifugation at 1000 *g* for 5 min. The cells were resuspended in Buffer A at a volume equal to that of the cell pellet and then frozen in liquid nitrogen. Cell pellets were allowed to thaw for 10 min in an ice/water bath. An equal volume of Buffer A was added and the sample was centrifuged at 14,000 *g* for 20 min in an Eppendorf microcentrifuge. The clear supernatant is referred to as cytosol and was used immediately in the immunoadsorption assay described in the following section.

Immunoadsorption assay

Cytosol, prepared from 40 to 60 million cells, was mixed with an equal volume of either the crude tissue culture supernatant containing antireceptor antibody (BuGR-2) or a 1:20 dilution (100 μg/ml) of the ascites fluid containing anti-hsp90 antibody (AC88). Tween-20 was added to give a final concentration of 0.1% and the antibody-cell extract mixture was continuously mixed by gentle rotation at 4°C for 1 h prior to addition of protein A-Sepharose. Protein A-Sepharose was suspended at 0.2 g/ml in buffer A and added in a volume equal to one-third of the volume of the antibody-cell extract mixture. The mixture was continuously mixed by gentle rotation for 30 min at 4°C. The protein A-Sepharose was then pelleted by centrifugation at 14,000 *g* in an Eppendorf microcentrifuge for 5 min at 4°C. The pellets were washed 5 times with either Buffer B or Buffer C, centrifuging each time in an Eppendorf microcentrifuge for 5 min at 4°C. The protein A-Sepharose pellets were suspended in 0.12 ml of the sample buffer for gel electrophoresis and heated at 100°C for 15 min. The samples were clarified by centrifugation in an Eppendorf microcentrifuge for 5 min at 25°C and subjected to gel electrophoresis.

Gel electrophoresis

The method of gel electrophoresis (SDS–PAGE) was modified after that of Laemmli[27] and has been described in detail [28]. Gels were stained with Coomassie Brilliant Blue and destained as described previously [28]. Gels were rinsed with water for 30 min and then soaked in 1 M sodium salicylate for 30 min prior to drying on to filter paper under vacuum. The position of size standards was marked on the dried gel using ink containing [^{35}S]methionine. Gels were exposed to Kodak XAR-5 film at −80°C for 12–24 h. The absorbance of bands on autoradiograms was measured using an LKB Ultroscan XL Laser Densitometer.

RESULTS AND DISCUSSION

Effect of RU486 on receptor–hsp90 complexes

S49.1 mouse lymphoma cells were pulse-labeled with [^{35}S]methionine for 30 min and then the radioactive label was chased by culturing the cells in medium supplemented with 1 mg/ml unlabeled methionine. Either no hormone (control), 0.1 μM RU486, or 0.1 μM dexamethasone were added to cells at the beginning of the chase. At 4 h into the chase, cells were ruptured by freezing and thawing in hypotonic buffer and labeled receptors were immunoadsorbed to protein A-Sepharose from the cytosol fraction using the BuGR2 monoclonal antireceptor antibody. Immunoadsorbed proteins were resolved by SDS–gel electrophoresis and analyzed by autoradiography.

Figure 1 shows that hsp90 coimmunoadsorbs with the glucocorticoid receptor from control cells. Molybdate was not present in the buffers employed in this experiment, demonstrating that receptor-hsp90 complexes can be recovered in the absence of molybdate. However, it should be noted that the buffer used to wash protein A-Sepharose pellets prior to gel electrophoresis contained a low concentration

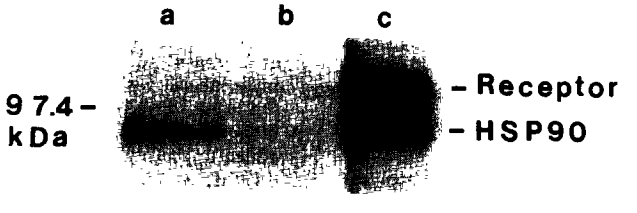

Fig. 1. Immunoadsorption of hsp90–receptor complexes. Cytosol was prepared from [^{35}S]methionine-labeled cells and incubated for 1 h at 4°C with AC88 antibody (lane a), no antibody (lane b) or BuGR2 antibody (lane c). Antibody–protein complexes were adsorbed to protein A-Sepharose and washed with low salt buffer in the absence of molybdate (Buffer C) prior to gel electrophoresis. An autoradiogram of the gel is shown.

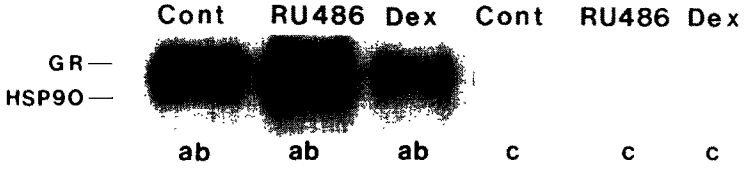

Fig. 2. Stabilization of hsp90–receptor complexes by RU486. S49.1 cells were pulse-labeled with [^{35}S]methionine and, at the beginning of the chase, were divided into three equal aliquots. One aliquot of cells was cultured in the absence of hormone (control). The second aliquot of cells was cultured in the presence of 0.1 μM RU486. The third aliquot of cells was cultured in the presence of 0.1 μM dexamethasone. At 4 h into the chase, cytosol was prepared from cells and labeled receptors were immunoadsorbed to protein A-Sepharose. Protein A-Sepharose pellets were washed with high salt buffer in the absence of molybdate (Buffer B) prior to gel electrophoresis. The autoradiogram shows the region of the gel opposite the 97.4 kDa standard. ab, antireceptor antibody; c, no antibody, control.

of salt (250 mM NaCl). We previously found this to be necessary in order to recover receptor–hsp90 complexes in the absence of molybdate, since receptor–hsp90 complexes are dissociated by a high salt concentration (400–500 mM NaCl) [26]. In Fig. 2, protein A-Sepharose pellets were washed with buffer containing 500 mM NaCl and hsp90 did not coimmunoadsorb with receptors from control cells. However, hsp90 did coimmunoadsorb with receptors from RU486-treated cells under the same high salt conditions, indicating that RU486 stabilizes receptor–hsp90 complexes against salt-induced dissociation. This result has been confirmed in three separate experiments and is consistent with earlier evidence that RU486 stabilizes the nonactivated, heterooligomeric form of the glucocorticoid receptor that contains hsp90 [11, 12]. It should be noted that RU486 was added to intact cells and receptor–hsp90 complexes were recovered immediately after cytosol preparation. Thus, it is likely that the stabilizing effect of RU486 on receptor–hsp90 complexes occurs intracellularly.

The results of Fig. 2 also indicate that RU486 and dexamethasone have different effects on the association between hsp90 and the glucocorticoid receptor. We and others have previously found that hsp90 dissociates from the glucocorticoid receptor when the receptor binds a glucocorticosteroid hormone such as dexamethasone [17, 19, 26]. The dissociation of hsp90 from the receptor appears to accompany conversion of the receptor from an inactive (non-DNA-binding) form to an active (DNA-binding) form [17, 19]. In contrast to dexamethasone, the binding of RU486 to the glucocorticoid receptor is not accompanied by dissociation of hsp90, indicating that RU486 stabilizes the inactive (non-DNA-binding) form of the glucocorticoid receptor.

The preceding experiments employed cells that were cultured in the presence of fetal calf serum. It is possible that the stabilizing effect of RU486 on receptor–hsp90 complexes might be due to competition between RU486 and endogenous cortisol for receptor binding, thus preventing cortisol induced dissociation of receptor–hsp90 complexes.

Alternatively, RU486 could directly stabilize the receptor–hsp90 complex through its interaction with the receptor. In Fig. 3, pulse-chase labeling experiments were performed using cells that were cultured in Phenol Red-free medium supplemented with serum that had been extracted with charcoal to remove endogenous cortisol. hsp90 did not coimmunoadsorb with receptors from control cells, indicating that receptor–hsp90 complexes were no more resistant to salt-induced dissociation in the absence of endogenous cortisol as in the presence of endogenous cortisol. hsp90 did coimmunoadsorb with receptors from RU486-treated cells, confirming that RU486 stabilizes receptor–hsp90 complexes against salt-induced dissociation. These results suggest that the stabilizing effect of RU486 on receptor–hsp90 complexes is likely to be a direct effect of the binding of RU486 to the receptor rather than an indirect effect resulting from displacement of endogenous cortisol from the receptor by RU486.

Effect of RU486 on nuclear localization

Figure 2 also indicates that RU486 differs from dexamethasone in terms of its effect on receptor localization in the cell. Dexamethasone, but not RU486, induces a quantum drop in the recovery of labeled receptors from the cytosol fraction when added to cells at the beginning of the chase. This

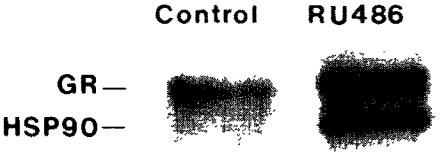

Fig. 3. Recovery of hsp90–receptor complexes from cells cultured under cortisol-deficient conditions. S49.1 cells were cultured in medium containing serum that had been charcoal treated to remove endogenous cortisol. Cells were pulse-labeled with [^{35}S]methionine and, at the beginning of the chase, were divided into two equal aliquots. No hormone was added to one aliquot (control) and RU486 was added to the other aliquot to give a final concentration of 0.1 μM. At 4 h into the chase, cytosol was prepared from cells and labeled receptors were immunoadsorbed to protein A-Sepharose using the BuGR2 antibody. Protein A-Sepharose pellets were washed with high salt buffer in the absence of molybdate (Buffer B) prior to gel electrophoresis. The autoradiogram shows the region of the gel opposite the 97.4 kDa standard.

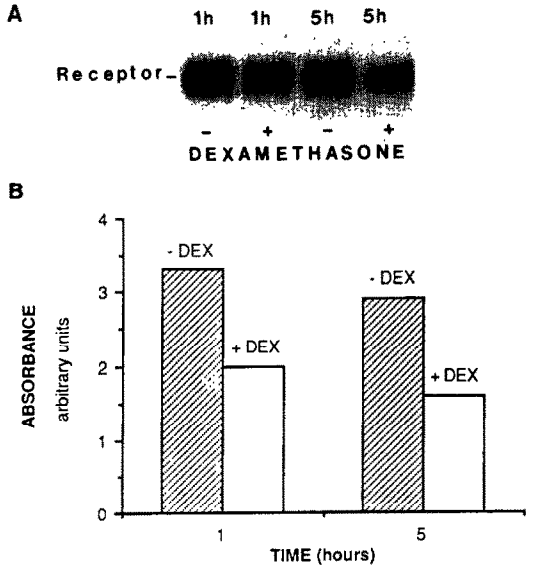

Fig. 4. Effect of dexamethasone on receptor recovery in S49.1 cells. S49.1 cells were pulse-labeled with [^{35}S]methionine and then cultured in the presence or absence of 0.1 μM dexamethasone (DEX) during the chase. At 1 and 5 h into the chase, cytosol was prepared from cells and labeled receptors were immunoadsorbed and subjected to SDS–PAGE. Gels were analyzed by autoradiography. *Panel A*: Region of the autoradiogram opposite the 97.4 kDa standard showing radiolabeled receptors recovered by immunoadsorption. *Panel B*: Absorbance of receptor bands shown in panel A, measured by scanning densitometry.

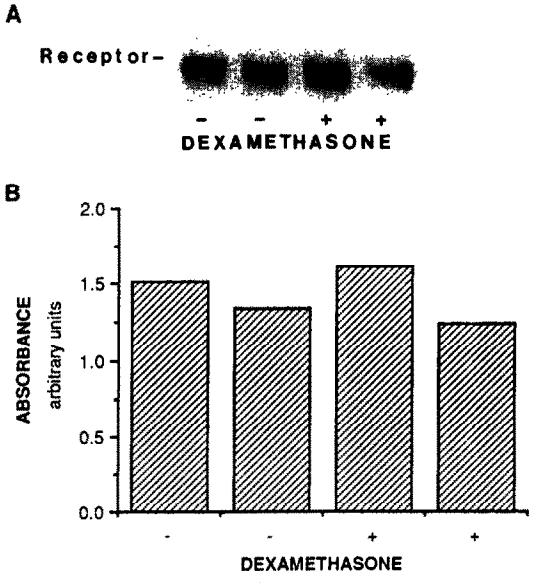

Fig. 5. Effect of dexamethasone on receptor recovery in S49.22r cells. S49.22r cells were pulse-labeled with [^{35}S]methionine and then cultured in the presence or absence of 0.1 μM dexamethasone during the chase. At 4 h into the chase, cytosol was prepared from duplicate sets of cells and labeled receptors were immunoadsorbed and subjected to SDS–PAGE. Gels were analyzed by autoradiography. *Panel A*: Region of the autoradiogram opposite the 97.4 kDa standard showing radiolabeled receptors recovered by immunoadsorption. *Panel B*: Absorbance of receptor bands shown in panel A, measured by scanning densitometry.

observation has been confirmed in 10 separate pulse-chase labeling experiments. For the following reasons, we believe that the dexamethasone-induced decrease in recovery of labeled receptors from cytosol is due to tight nuclear binding of receptors occupied by dexamethasone. First, as shown in Fig. 4, a drop in recovery of labeled receptors is detected within 1 h of adding dexamethasone to cells, consistent with earlier evidence that receptors localize to the nucleus within minutes of adding dexamethasone to cells [29]. Second, as shown in Fig. 5, dexamethasone does not decrease the recovery of receptors from cytosol of S49.22r cells, which are variants of the S49.1 cell line in which receptors do not undergo nuclear localization due to a single amino acid substitution in the DNA-binding region [30, 31].

Since RU486, unlike dexamethasone, does not decrease the recovery of receptors from the cytosol fraction, we conclude that receptors occupied by RU486 either do not translocate into the nucleus, or if they do enter the nucleus they are not tightly bound so that they are released into the cytosol fraction when cells are ruptured. In this regard, it is important to note that the cytosol preparation procedure employed here does not yield intact nuclei. Thus, it is possible that RU486–receptor complexes translocate into the nucleus, as suggested by earlier autoradiographic and immunocytochemical studies [8, 9], but do not bind as tightly to chromatin as dexamethasone–receptor complexes. This conclusion is consistent with earlier evidence that RU486–receptor complexes do not bind as tightly as dexamethasone–receptor complexes to DNA *in vitro* [2–7].

General discussion

The results of the present study indicate that the antiglucocorticoid hormone, RU486, stabilizes the association between the glucocorticoid receptor and hsp90. Although a physiologic role of hsp90 in glucocorticoid receptor function has not been established, there is strong evidence that the receptor–hsp90 complex corresponds to the nonactivated (non-DNA-binding) form of the glucocorticoid receptor and that receptor activation to a DNA-binding form is accompanied by dissociation of hsp90 [17, 19]. It is probable, therefore, that the antiglucocorticoid activity of RU486 derives from its ability to stabilize the association between the receptor and hsp90, thus preventing receptor activation *in vivo*.

The results are also consistent with the postulate that receptors occupied by RU486 are not bound as tightly in the nucleus as receptors occupied by dexamethasone. It is possible that the association of the receptor with hsp90 in the presence of RU486 may contribute to the decreased affinity of RU486–receptor complexes for the nucleus. Thus, hsp90 may block the DNA-binding site on the receptor or alter receptor conformation in such a way as to decrease the affinity of the receptor for DNA.

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