Developmental and Hormonal Regulation of Glucocorticoid Receptor Messenger RNA in the Rat

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Abstract

The biological action of glucocorticoids is dependent upon tissue-specific levels of the glucocorticoid receptor (GR). During stress, the hypothalamic-pituitary-adrenal axis is stimulated, and high levels of glucocorticoids circulate. This axis is modulated by negative feedback by glucocorticoids, which inhibit hypothalamic and pituitary hormone secretion and downregulate GR gene expression. To study the developmental tissue-specific regulation of the GR, we measured the relative concentration of GR mRNA in fetal, neonatal, adult, and aged rats and examined the effects of dexamethasone on GR gene expression. Three different tissue-specific developmental patterns of GR mRNA accumulation were found. In addition, there was an age-dependent tissue-specific pattern in the feedback regulation of GR mRNA by glucocorticoids. In the fetus and neonate, GR mRNA abundance was not regulated by circulating glucocorticoids. The adult pattern of glucocorticoid feedback inhibition of GR mRNA expression appeared between 2 and 7 d of life in liver, and after 7 but before 14 d of age in brain. The GR was biologically active in the 2-d-old neonate, however, since dexamethasone enhanced gene expression of angiotensinogen, which is another glucocorticoid responsive gene. These data demonstrate that the GR gene is regulated by both developmental and tissue-specific factors, and provide another molecular basis for ontogenic variations in the hypothalamic-pituitary-adrenal response to stress.

Introduction

Glucocorticoid homeostasis is maintained by the tightly regulated hypothalamic-pituitary-adrenal axis. Under the influence of corticotropin-releasing hormone (CRH) and arginine vasopressin, ACTH is released in a pulsatile pattern with a characteristic diurnal rhythm (1). ACTH induces the release of adrenal corticosteroids, which then interact with specific receptors in the brain and pituitary gland to inhibit further release of both CRH and ACTH (2–4). This carefully maintained balance is interrupted during periods of stress when high circulating levels of glucocorticoids are needed for survival.

Two corticosteroid receptors have been characterized by differential binding studies using radiolabeled steroid ligands. The type I, or mineralocorticoid receptor, has been studied most extensively in the kidney, gut and salivary glands, where it mediates sodium retention (5, 6). In the brain, the type I receptor has been shown to influence salt appetite (7). The type II, or glucocorticoid receptor (GR), is found in almost every cell in the body and it subserves a multitude of physiologic functions ranging from glycogen deposition in the liver to inhibition of lymphocyte function (8, 9). The type II receptor is also the presumed mediator of glucocorticoid negative feedback in the central nervous system and pituitary (2–4). Glucocorticoids can modulate their own action not only by inhibition of the hypothalamic-pituitary axis, but also by altering the concentration of their own receptors. In the adult vertebrate, glucocorticoids regulate the GR by a negative feedback downregulation of receptor number (8, 9). Using an in vitro paradigm, Rosewicz and colleagues (10) showed that this downregulation may be partially controlled at the transcriptional level, and we recently demonstrated that glucocorticoid administration reduced GR mRNA levels in adult rats (11). This further refinement in the glucocorticoid negative feedback process could theoretically act to brake excessive inhibition of the CRH-ACTH network, allowing the higher levels of glucocorticoids needed during stress.

Developmental changes also influence the hypothalamic-pituitary-adrenal axis. In the early neonatal period in the rat, circulating corticosteroid and transcortin levels are low (12), and the adrenocortical response to stress is greatly reduced (13, 14). It has been suggested that this quiescent, stress nonresponsive (SNR) period (∼5–14 d of age in the rat) protects the CNS from the potentially deleterious effects of high concentrations of glucocorticoids (15). Several studies have demonstrated that multiple factors may contribute to the SNR period including: (a) the smaller size and diminished synthetic capacity of the neonatal adrenal gland; (b) the decreased hypothalamic content of CRH and the consequent decrease in ACTH release during stress; and (c) a changing number of glucocorticoid receptors (16). After the SNR, there is an increase in circulating glucocorticoid and transcortin levels (14–21 d of age in the rat) as well as an increase in GR number (12). The fact that both glucocorticoid levels and GR number increase in parallel during this phase of development indicates that in the neonate, glucocorticoids do not regulate the GR by the classic feedback downregulatory mechanism (10, 11) seen in the adult. These data suggest that the GR may not be a glucocorti-
coid-responsive gene in all tissues throughout the animal's lifespan. The purpose of this study is to learn how GR mRNA accumulation changes during development and to examine the role of glucocorticoids in the regulation of GR gene expression.

Previous investigations of the ontogeny and regulation of the GR have been hampered by methodologic difficulties. Studies that use radiolabeled ligand binding alone often do not completely distinguish between the type I and type II corticosteroid receptors, because natural and synthetic steroids may bind to both receptors and because there may be incomplete exchange of labeled ligand for endogenous ligand. This latter problem is particularly important in those studies where endogenous corticosteroid levels are changing. In this study, these difficulties have been avoided by using a GR-specific cRNA probe to characterize the developmental regulation of GR gene expression in rat brain, kidney, liver, and lung obtained from fetal, neonatal, adult, and aged animals. The ability of glucocorticoids to regulate GR mRNA in several tissues at these developmental stages has also been examined. Finally, the developmental expression and regulation of GR mRNA by glucocorticoids has been compared to that of a second glucocorticoid responsive gene, angiotensinogen (AT), to examine the biologic function of the GR in a separate gene system.

Methods

Animals. Male Sprague-Dawley rats (Simenson Laboratories, Gilroy, CA) were maintained according to Stanford University guidelines on ad lib. rat chow and tap water with a 12-h light/dark cycle. All animals were killed by decapitation and the tissues were immediately removed, frozen in liquid nitrogen, and stored at −80°C before RNA isolation. Fetal tissues were obtained from timed-pregnant rats (Simenson). For each age analyzed, a total of 3–12 rat organs were pooled before RNA isolation.

RNA isolation. Total cellular RNA was isolated from frozen tissue as previously described (17) or by the method of Chirgwin et al. (18). Briefly, tissues were homogenized in the presence of 4 M guanidine thiocyanate (Fluka Chemical Corp., New York) and 1 M 2-mercaptetoethanol, and allowed to stand at room temperature for a minimum of 3 h. CsCl was added to a final concentration of 1.5 M, and the solution was layered over a 5.7-M CsCl cushion containing 6 mM 2-mercaptoethanol. The RNA was pelleted by ultracentrifugation at 35,000 rpm for 20 h. RNA pellets were dissolved in sterile water, quantitated by standard ultraviolet absorbance, and analyzed for RNA integrity by agarose gel electrophoresis (18).

Synthesis of probes. The GR cRNA probe utilized in these experiments was the cRNA transcript of the 3' nontranslated, 2.2-kb Xba-Pst I fragment of pRM16 rat glucocorticoid receptor clone (19) inserted into pSP65 Riboprobe in the anti-sense orientation (a kind gift of Keith Yamamoto, University of California, San Francisco). Its specificity for GR mRNA has previously been demonstrated (11). The AT cRNA probe was synthesized from a pGem-3 Pvu II linearized 3' Pst I fragment (17). The 32P-labeled RNA transcripts of these probes were synthesized to an average specific activity of 1 × 106 cpm/μg using SP6 RNA polymerase as previously described (17).

A β-actin plasmid (kind gift of Lawrence Kedes) was nick translated with [32P]dCTP and [32P]dATP using standard techniques (18).

RNA quantification. Total GR or AT mRNA was quantitated by slot blot hybridization as previously described (17). Briefly, total RNA was serially diluted (4.0–1.0 μg/sample) in a 400-μl solution containing 6.15 M formaldehyde and 10× SSC. The RNA was denatured at 65°C for 20 min and then immobilized on 0.1-μm nitrocellulose filters (Schleicher & Schuell, Keene, NH) by slow vacuum filtration. The wells were rinsed with 400 μl of 10× SSC and the RNA fixed to the filters by baking at 80°C in vacuo for 2 h. Prehybridization and hybridization procedures were performed at 65°C for GR and at 60°C for AT in 50% formamide, 3× SSC, 10× Denhardt's solution, 20 mM Tris (pH 7.6), 10 mM EDTA (pH 8.0), 200 μg/ml sheared salmon sperm DNA, and 0.2% SDS with the hybridization buffer containing 2.5–3.0 × 105 cpm of [32P]cRNA probe. After hybridization, the membranes were rinsed with 2× SSC (24°C) followed by sequential 65°C (GR) or 60°C (AT) washes in 2× SSC, 0.2% SDS, and 0.2× SSC, 0.2% SDS. To ensure accurate RNA loading on all slot blots, duplicate filters were hybridized with actin (42°C in a modified hybridization solution containing 5× SSC). The filters were washed at 50°C. In addition, control experiments comparing actin hybridized to duplicate filters vs. stripped reused filters revealed the same pattern of actin mRNA expression. The only difference was that each round of stripping appeared to result in an equal loss of ~5–10% of the RNA bound. Autoradiographs were obtained by exposure to Kodak XAR-5 film with an intensifying screen at ~80°C for 12–96 h. The autoradiographs were scanned with a laser densitometer (2202 Ultron Scan; LKB Instruments, Inc., Piscataway, NJ) to quantify the relative amount of hybridized probe. Control experiments confirmed quantitative binding of the RNA to the filters. Multiple exposure times were obtained to ensure that the autoradiograph was within the linear range for quantitative scanning.

Dexamethasone regulation of GR and AT mRNA. For the time course study, total RNA was isolated from adult rat livers and brains (three animals/time point) at 2, 4, 6, 8, 15, and 24 h after a single intraperitoneal injection of dexamethasone (DEX, 7 mg/kg). In subsequent experiments, total RNA was isolated from neonatal or adult rat tissues 6 h after the animals received a single injection of vehicle (control animals), or of DEX at "stress" (150 μg/kg i.p.) or "pharmacologic" (7 mg/kg i.p.) (20) doses. Similarly, fetal RNA was obtained 6 h after the administration of vehicle or DEX (150 μg/kg or 7 mg/kg i.p.) to the pregnant female rats. The relative abundance of the GR, AT, and actin mRNA was measured by slot blot hybridization and laser densitometric measurements as described above.

Northern blot hybridization. Total RNA was isolated (17), denatured with glyoxal, and electrophoresed through a 0.9% agarose gel. The RNA was transferred and fixed to Hybond-N (Amersham Corp.) (17). The filter was sequentially hybridized with a GR cRNA probe and an actin cDNA probe.

Statistical analysis of data. Student's t test was used to analyze the changes in GR mRNA after dexamethasone treatment.

Results

Tissue-specific developmental regulation of GR mRNA. Total RNA was isolated from fetal, neonatal, adult, and aged rat tissues. The relative abundance of GR mRNA was then quantitated by slot blot hybridization with densitometric scanning of the resulting autoradiographs (Fig. 1). The relative tissue levels of GR mRNA in adult rats were previously published (11) and cannot be obtained from Fig. 1 because variable exposure times were used to obtain optimal autoradiographs for densitometric scanning and quantitation of the developmental changes in GR mRNA within a given tissue.

Three different developmental patterns of GR mRNA accumulation were found. During late fetal development (gestational age 18 and 20 d), GR mRNA abundance in the brain decreased 40% when compared to earlier fetal ages (days 15 and 17) (Fig. 1). After birth, the brain GR mRNA levels gradually increased, reaching adult levels by 3 wk of age. The adult brain GR mRNA levels were approximately twofold greater than those found in the newborn pup. In contrast, actin mRNA abundance gradually declined with age, as previously described by Bond and Farmer (21).
A similar developmental change in the expression of the GR gene was noted in the fetus (Fig. 1). Again, the GR mRNA levels decreased by ~50% from a peak at gestational day 15 to a nadir at gestational day 20. These levels increased only slightly above fetal levels during the first 2 wk postpartum. Over the subsequent months, the abundance of GR mRNA gradually increased reaching a plateau at 3 mo and a maximum by 9 mo of age. The GR mRNA was 2.5-fold more abundant in the aged rat liver when compared to fetal GR mRNA levels. During the time of increase in GR mRNA, actin mRNA does not substantially change.

In contrast, different patterns of GR mRNA regulation were detected in the kidney and lung (Fig. 1). A gradual 30% decrease in kidney GR mRNA occurred during the first 5 d postpartum, followed by a twofold increase by day 14. Over the next 1–6 mo, the GR mRNA decreased to the final adult value. The resulting GR mRNA abundance in the adult kidney was only 10% of the 14-d peak value and 20% of early neonatal levels. We had previously demonstrated that the lung had the highest levels of GR mRNA of all tissues examined (11). There was no substantial change in lung GR mRNA levels throughout development. In both of these tissues, there was little change in actin mRNA.

Thus, the developmental pattern of the GR gene expression appeared to be tissue specific. Moreover, from 9 to 20 mo of age, no further alterations in GR mRNA abundance was detected in any of the four tissues examined.

**DEX regulation of GR mRNA in brain and liver of fetal, neonatal, adult, and aged rats.** The increase in the brain and liver GR mRNA levels occurred at a time when plasma concentrations of both free and bound corticosterone were also increasing (12), suggesting that the classic adult pattern of negative feedback regulation of the GR may not be operant in the fetus or neonate. To test this hypothesis, we determined the time course of glucocorticoid regulation of GR mRNA levels. 8-wk-old rats received a single injection of DEX (7 mg/kg i.p.). Total RNA was isolated at 2, 4, 6, 8, 15, and 24 h after the injection and the relative abundance of GR mRNA was determined by slot blot hybridization (Fig. 2). Both the liver and brain showed maximal decreases in the GR mRNA 4–8 h after the animals received the DEX, and this decrease persisted for at least 24 h after the injection. As depicted in the northern blot hybridization (Fig. 3), the decrease in GR mRNA after DEX administration did not substantially affect the levels of actin mRNA which was used as a control to ensure equal RNA loading. In subsequent experiments, we compared the effect of glucocorticoid excess on GR mRNA in the brain and liver of fetal, neonatal, and adult rats 6 h after the administration of DEX. The neonatal, adult, or pregnant animals were treated with DEX to mimic the level of glucocorticoid elicited by a maximal “stress” (150 μg/kg) or to provide a pharmacologic level of glucocorticoids (7 mg/kg) (20). No change in brain or liver GR mRNA levels were observed when 20-d fetal or 2-d neonatal rats were exposed to DEX (Fig. 4). At 2 wk of age, the classic negative feedback downregulation of the GR mRNA was apparent in both organs, with a decrease of GR mRNA levels of up to 45–55% after DEX treatment. Both tissues demonstrated greater decreases in GR mRNA when pharmacologic injection of DEX was obtained. A 50% decrease in GR mRNA is observed in lane 2 while the actin mRNA is not substantially altered.
logic doses of DEX were utilized. The decreased responsiveness to DEX in the fetal organs was difficult to evaluate extensively since the concentration of circulating DEX achieved in the fetus is unknown. Therefore, subsequent experiments examined the lack of response to DEX in the neonatal animals in greater detail.

To examine the liver and brain developmental pattern more closely, the regulation of the GR mRNA was studied in 1- and 6-wk-old rats and for statistical analysis, the tissues were not pooled. No decrease in brain GR mRNA was found after DEX exposure in the 1-wk-old rat brain. However, a 50% reduction was seen in brains of 6-wk-old DEX treated rats when compared with age matched controls (*P < 0.05) (Fig. 5 A). In contrast, DEX treatment resulted in a 40 and 55% decrease in liver GR mRNA levels of 1- and 6-wk-old rats, respectively (*P < 0.05) (Fig. 5 B).

DEX regulation of GR mRNA in the liver and brain of neonatal and adult rats. The above results suggested that DEX failed to regulate GR mRNA content in the early neonate. Since both the GR mRNA and protein are present in neonatal rats, one explanation for the failure of DEX to regulate the GR mRNA at this point in development is the possibility that there is a general defect in glucocorticoid hormone binding or in the posthormone binding steps that are necessary for glucocorticoid responsive gene activation. To test this hypothesis, we investigated the ability of glucocorticoids to modulate AT mRNA, a known glucocorticoid responsive gene. We previously reported that in the adult rat, DEX treatment causes a 300% increase in AT mRNA in the liver and a 60% increase in the brain (17). The same 20-d fetal and 2-d old neonatal liver and brain total RNA specimens which had been studied with the GR probe were slot blotted and hybridized with an AT cRNA probe and the autoradiographs were quantitated by laser densitometric scanning. While DEX treatment (7 mg/kg) did not change AT mRNA abundance in the fetal rats, a 120% increase in AT mRNA in the liver and a 56% increase in AT mRNA in brain were observed in the 2-d-old animals (data not shown). These studies demonstrate that the glucocorticoid-GR complex is biologically functional in early neonatal life.

Discussion

An intact hypothalamic-pituitary-adrenal stress response is crucial for the survival of adult vertebrates. During the early postnatal period, however, the rat pup's capacity to respond to environmental stresses by increasing corticosteroid concentration or inducing glucocorticoid responsive genes is greatly diminished (13–15). The physiologic advantage of this altered response, and the mechanism by which it is achieved remains uncertain. An intact hypothalamic-pituitary-adrenal axis requires (a) the secretion of CRH and other releasing hormones from the hypothalamus; (b) the subsequent release of ACTH from the pituitary gland; (c) glucocorticoid synthesis in and secretion from the adrenal gland in response to the ACTH; and finally, (d) the binding of the glucocorticoid to its receptor resulting in a biologic response. During development, a variety of physiological modifications occur at each of these steps. It is possible, for example, that the SNR is associated with changes in GR levels or with changes in the ability of glucocorticoids to alter gene expression. However, experimental proof for these associations is lacking due to limitations of the GR binding assay, including the need for a complete exchange of the radiolabeled for the endogenous ligand, the use of adrenalectomized animals, and the possible subcellular redistribution of the glucocorticoid receptors which may complicate the accuracy of receptor quantitation. These problems may also explain the lack of consensus in the literature on the times at which adult levels of GR are attained (22). While these studies all demonstrate that lower GR concentrations are present in the neonate and that identical GR-ligand binding affinities are found throughout development, the binding assays cannot evaluate whether the receptor-ligand complex is biologically active.

The recent cloning of the rat GR (19) gene and the demonstration that glucocorticoids exert negative feedback on the GR gene by transcripational and posttranslational regulatory mechanisms (23) now provide the tools needed to investigate the ontogenic regulation of the GR gene and the biologic effectiveness of glucocorticoids on GR mRNA regulation. Our approach differs in important ways from previous studies. First, the GR cRNA probe has been demonstrated to be specific for the GR gene. Unlike some binding studies, therefore, the presence of mineralocorticoid receptors will not affect the quantitation of the GR gene product. Because we are measuring mRNA levels, there is no need to be concerned with possible
developmental changes in serum steroid concentrations that can complicate the labeled ligand exchange assay. Similarly, there is no need to adrenalectomize the animals. This is important since adrenalectomy has been shown to increase the half-life of the GR (24). Finally, changes in mRNA levels after glucocorticoid exposure can be determined rapidly, since the GR gene is transcriptionally regulated by glucocorticoids (23) and changes in GR mRNA can be detected ≈ 24 h before subsequent detection of changes in receptor number are found using conventional binding assays (25). However, with this approach we cannot determine if the changes in mRNA are due to changes in transcription or to changes in mRNA half-life. Furthermore, while previous data in adult rats suggest that GR mRNA levels reflect changes in GR protein (11), we did not measure the GR protein levels directly, and we cannot rule out developmental differences in the translational control of the GR protein.

We have demonstrated that the ontogenic regulation of the GR mRNA levels was tissue specific by utilizing the GR cRNA probe. We have previously shown that the lung had the highest levels of GR mRNA with the relative levels in the other tissues of: liver, 50%; brain, 55%; and kidney, 43% (11). Throughout neonatal development and aging, the lung demonstrated high invariant levels of GR mRNA.

The liver and brain showed a second type of GR mRNA developmental pattern. In these tissues the GR mRNA levels decreased just before birth. Postpartum, the liver GR mRNA levels remained at constant low levels for the first 2 wk, increased to a plateau at 3 mo of age, and reached a maximum by 9 mo. Binding assays have shown that the rat liver GR concentration reached a maximum anywhere from 2 d to 3 wk (17) of age. This wide range in ages may reflect the difficulty of using exchange binding assays to quantitate the GR during this period of increasing free and bound corticosteroid concentrations. In the brain, GR mRNA levels remained low for the first postpartum week and subsequently rose and achieved the final adult levels by 3 wk of age. This developmental pattern of CNS GR mRNA agrees with the receptor binding data of Clayton et al. (26) and Olpe and McEwen (27).

In the kidney, the GR mRNA levels increased from days 7 to 14 and then declined to final adult basal levels by ~ 3–6 mo of age. An initial increase in GR has not been reported in binding studies, but the subsequent decrease in GR mRNA observed in this study is consistent with the published studies of Sharma et al. (28) and Aperia et al. (29) comparing GR binding in 20-d and 6–8-wk adult rats.

Glucocorticoids are known to affect the growth and development of a wide range of eukaryotic cell types. These effects are mediated by the translocation of the GR-ligand complex to the cell nucleus (30), and there is a direct correlation between GR concentration and glucocorticoid responsiveness (31). More recent studies utilizing cotransfection of the GR gene and reporter genes into GR negative cells have shown that the magnitude of the transcriptional response of the reporter gene was proportional to the number of GR gene copies (32). However, glucocorticoid hormone action may be modulated by the fact that GR number (33), GR half-life (10), and GR mRNA levels (11, 34) decrease with increasing concentrations of glucocorticoids. The importance of this regulation for cellular homeostasis is underscored by the recent findings of Eisen et al. (35). They showed that while excess glucocorticoids downregulate both GR mRNA and GR receptor number in the normal human T cell, in the human T cell line, CEM-C7, both the GR protein and its mRNA increased significantly with exposure to glucocorticoid, and that this treatment ultimately resulted in cell death.

The ontogenic regulation of the GR gene needs to be placed in the context of changing circulating glucocorticoid levels in the developing animal. Beginning in the late fetal period in the rat, the basal circulating levels of corticosterone are high (12). Immediately postpartum however, the corticosterone levels decline dramatically and remain low for the first 2 wk. Also during this time, the pups respond minimally to stress, maintaining unperturbably low corticosterone levels (13–15). During the third week of life, the free and bound corticosterone levels rapidly increase, peaking above adult levels; the levels gradually decline to the final adult corticosterone concentrations during the fourth week of life (12). The greatest increases in receptor mRNA levels (see Fig. 1) and in receptor protein number (12) in kidney, brain, and liver occur during a time when glucocorticoid levels are rising and when one might therefore expect to find feedback downregulation of receptor mRNA and number. In addition, the hypothalamic-pituitary-adrenal axis is not suppressed by endogenous glucocorticoids during the SNP. Meaney et al. (36) were unable to inhibit endogenous corticosterone secretion with dexamethasone in neonatal rats <15 d old. These unexpected results suggest that there may be age related differences in the biologic action and/or gene regulation of the GR. Salky and Koch (37) recently demonstrated that in extracts from the 2–6-d-old neonate, only 20% of the pituitary cytoplasmic [3H]DEX-receptor complexes translocated to the nucleus while 100% translocated in adult preparations. The adult translocation rate was also found in 10-d-old pituitary preparations or when adult pituitary cell cytoplasm was combined with pituitary nuclei from 2-d-old animals.

The results of our studies do not completely support the translocation hypothesis. We have shown that while pharmacologic doses of DEX failed to decrease GR mRNA in the 2-d-old rat, AT mRNA increased in the liver and in the brains of the same animals. These data suggest that the GR-ligand complex in 2-d-old rats was biologically functional and that other factors may be specifically influencing the feedback regulation of the GR mRNA in early life. Alternatively, the lack of GR downregulation by glucocorticoids in the neonatal period may reflect a biological requirement for the presence of a minimal level of GR mRNA. In the fetus, neither GR nor AT mRNAs responded to dexamethasone treatment. This failure to downregulate may reflect either an immature, nonfunctional GR-complex, increased maternal-fetal metabolism of dexamethasone, or the failure of adequate concentrations of dexamethasone to cross the placenta. Further studies will be necessary to differentiate between these possibilities.

In conclusion, we have demonstrated three different tissue-specific developmental patterns of GR mRNA accumulation. There was both an age-dependent and a tissue-specific pattern in the feedback regulation of GR gene expression by glucocorticoids. In the fetus and the neonate, GR mRNA abundance was not regulated by circulating glucocorticoids and the change to the adult pattern of ligand feedback downregulation of the GR mRNA occurred between 2 d and 2 wk of age. In addition, we have demonstrated that the GR-ligand complex, as determined by the induction of AT mRNA, was biologically functional in the early neonate. Thus, the
age-dependent alterations seen in the feedback regulation of the GR gene cannot be explained by an immature or non-functioning GR-ligand complex or by the lack of translocation of this complex to the nucleus.

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