Selective Activation of the Glucocorticoid Receptor by Steroid Antagonists in Human Breast Cancer and Osteosarcoma Cells*

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Steroid hormones regulate the transcription of numerous genes via high affinity receptors that act in concert with chromatin remodeling complexes, coactivators and corepressors. We have compared the activities of a variety of glucocorticoid receptor (GR) antagonists in breast cancer and osteosarcoma cell lines engineered to stably maintain the mouse mammary tumor virus promoter. In both cell types, GR activation by dexamethasone occurs via the disruption of mouse mammary tumor virus chromatin structure and the recruitment of receptor coactivator proteins. However, when challenged with a variety of antagonists the GR displays differential ability to activate transcription within the two cell types. For the breast cancer cells, the antagonists fail to activate the promoter and do not promote the association of the GR with either remodeling or coactivator proteins. In contrast, in osteosarcoma cells, the antiglucocorticoids, RU486 and RU43044, exhibit partial agonist activity. The capacity of these antagonists to stimulate transcription in the osteosarcoma cells is reflected in the ability of the RU486-bound receptor to remodel chromatin and associate with chromatin-remodeling proteins. Similarly, the observation that the RU486-bound receptor does not fully activate transcription is consistent with its inability to recruit receptor coactivator proteins.

Clinically, steroids and steroid antagonists are widely used in the treatment of endocrine disorders, cardiovascular disease, and cancers, including those of the breast and ovary (1). Steroids regulate growth and development through binding to a superfamily of high affinity steroid receptors (SR) that regulate the transcription of target genes (2). Upon binding ligand the SR undergoes a conformational change that allows the receptor to dimerize and bind to the hormone response element within target genes (2). This multistep pathway, which ultimately results in changes in gene transcription, can be manipulated pharmacologically by steroid hormone antagonists. In the case of the glucocorticoid receptor (GR), antiglucocorticoids have been shown to either block the capacity of the GR to interact with the hormone response element or interfere with the subsequent processes linked to transcriptional activation (3, 4).

Differences in the activity of hormones and antihormones may reflect the ability with which the cellular transcriptional machinery discriminates between structurally distinct receptor conformations at the carboxyl-terminal transcriptional activation domain (5–7). This region of the receptor is thought to form a protein interaction surface for steroid receptor coactivators (8, 9). Accordingly, interactions between steroid antagonists and the receptor would block the coactivator recruitment site, and this may then prevent transactivation.

Steroid antagonists exhibit a range of activity between “pure” antagonists that efficiently antagonize receptor function and “mixed” antagonists that may selectively stimulate receptor action depending on cell type and/or promoter context (10, 11). For example, with the estrogen receptor, ICI 164 384 is a pure antiestrogen, and trans-4-hydroxytamoxifen is a mixed agonist (12). Similarly, a class of progesterone receptor (PR) antagonists have been shown to function as mixed agonists (13). These include compounds such as RU486 that induce a conformational change in the PR that is different from that induced by agonists such as progesterone or antagonists such as ZK98299 (13, 14). Other studies suggest that the extreme carboxyl terminus of the PR may bind a corepressor that normally suppresses RU486 agonist activity resulting in partial agonist activity (15). Alternatively, the partial agonist activity of RU486 is enhanced in the presence of 8-bromo-cyclic AMP (10, 11). Thus multiple factors including receptor structure, corepressor expression, and activation of protein kinase A signaling may influence the transactivation properties of an antagonist-bound receptor.

Transactivation of steroid-responsive genes may also be regulated by the chromatin structure of the promoter (16, 17). In eukaryotic cells the DNA is intimately associated with histone and non-histone proteins, and the architecture of the DNA as chromatin can modulate gene expression (18). In general, the packaging of DNA into nucleosomes prevents the access of tumor virus; PAGE, polyacrylamide gel electrophoresis; Dex, dexamethasone; NF1, nuclear factor 1; BAFs, hBRG1-associated factors.
transcription factors to their binding sites and has a repressive effect on transcription (18, 19). Indeed, hormone-induced chromatin remodeling is a hallmark of activated transcription for steroid-regulated genes (17). The mouse mammary tumor virus (MMTV) promoter is a well defined model system to study glucocorticoid receptor activation of transcription (20). When stably introduced into cells, the MMTV promoter reproducibly acquires a phased array of nucleosomes. Glucocorticoid treatment induces remodeling of the chromatin, the binding of transcription factors, and concomitant transcriptional activation (21, 22).

In this study we examined the ability of antiglucocorticoids to induce transcription, chromatin remodeling, and transcription factor binding at the MMTV promoter in breast cancer and osteosarcoma cell lines. The antiglucocorticoids ORG31710 (Org) and ZK98299 (ZK98) displayed no agonist activity in either breast or osteosarcoma cells. In contrast, RU486 and RU43044 (RU43) exhibited partial agonist activity in osteosarcoma cells but not in breast cancer cells. This partial agonist activity of RU486 was consistent with its ability to induce GR-mediated chromatin remodeling and transcription factor loading and reflected the capacity of the RU486-bound receptor to recruit components of the chromatin remodeling complex to the promoter in osteosarcoma cells.

MATERIALS AND METHODS

Cell Lines—To generate a cell line expressing the MMTV-luciferase promoter, human osteosarcoma cell line ectopically expressing wild type GR, U2OS-GR, was cotransfected with pLTRLUC and a puromycin resistance plasmid, pGIPuro, in a ratio of 10:1 by LipofectAMINE (Life Technologies, Inc.) as described previously (4, 23). Stable transfectants were selected in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 500 µg/ml G418, and 1 µg/ml puromycin. Clonal derivatives were transferred to 6-well plates, grown to confluence, and screened for the presence of MMTV-luciferase by polymerase chain reaction. The clonal derivative UL3 was derived from U2OS-GR. T47D/A1-2 (A1-2) cells are human T47D breast cancer cells that were stably transfected with a GR expression plasmid and an MMTV-luciferase plasmid (24). The A1-2 cells were grown at 37°C with 5% CO2 in modified Eagle’s medium containing 10% fetal bovine serum.

Immunoprecipitation and Immunoblotting—Immunoprecipitation analysis was performed as previously (4). The washed immune complexes were resuspended in 2x SDS-PAGE loading buffer and released by boiling for 5 min. For immunoblot analysis, proteins were resuspended in loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and detected by Western blotting.

RESULTS

Antiglucocorticoids Exhibit Agonist Activity in Osteosarcoma Cells but Not in Breast Cancer Cells—Transcriptional activation from the MMTV promoter has been examined in a variety of mouse and human breast cancer cell lines (20). To examine MMTV activation by glucocorticoids and antiglucocorticoids in a second endocrine tissue, bone-derived osteosarcoma cells, stable cell lines were generated from U2OS-GR osteosarcoma cells (23) and compared with T47D/A1-2 breast cancer cells. U2OS-GR cells were stably transfected with pLTRLUC, which contains the full-length MMTV promoter driving the luciferase reporter gene. From the U2OS-GR clones positive for the MMTV plasmid and responsive to hormone, one of the clones (UL3) was selected for a detailed mechanistic characterization of the hormone response.

Steroid receptor antagonists have been used extensively to dissect receptor mechanisms of action (28, 29). RU486 is one of the most extensively studied and clinically important hormone antagonist (30, 31). Although it binds both the GR and the progesterone receptor (PR) with high affinity and promotes at least partial transformation of the receptor to active form, RU486 also possesses potent antiglucocorticoid and antiprogestin activities (30, 31). RU486 and another antiglucocorticoid Org31710 (Org) have been classified as type II antiprogestins.

\[\text{RU43044 (RU43)} \]

\[\text{ZK98299 (ZK98)} \]

\[\text{RU43044 (RU43)} \]

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Fig. 2. RU486 induces GR-mediated restriction enzyme hypersensitivity in UL3 but not A1-2 cells. The schematic indicates the cleavage sites for the restriction enzymes HaeIII, BamHI, and SstI as well as the position of the oligonucleotide used for polymerase chain reaction amplification (OJ). A. UL3 cells were untreated (C) or treated with either dexamethasone (D) (10^{-7} M) or RU486 (RU) (10^{-7} M) or ZK98299 (ZK) (10^{-7} M) for 1 h. Nuclei were isolated and digested with SstI for 15 min at 30 °C. After purification, genomic DNA was digested with HaeIII, and 10 μg of each sample was amplified using Taq polymerase and a 32P-labeled single-stranded primer specific to the MMTV promoter. Purified extension products were analyzed on a 8% polyacrylamide denaturing gel and exposed to Kodak X-Omat Blue film at -80 °C. B. A1-2 cells were treated as in A. After purification genomic DNA was digested with BamHI and analyzed as in A.

(32, 33). In contrast the compound ZK98299 (ZK98), although structurally related to RU486, has been classically defined as a type I antiprogestin that does not promote conversion of the PR to a DNA-binding form in vitro (32, 33). Previous analysis of the mechanism of action of ZK98299 within in vivo systems has led to divergent data (3, 34, 35). Although developed to possess weaker antiglucocorticoid activity than RU486, ZK98299 and Org31710 act as antiglucocorticoids at high concentrations (3, 36, 37). The antiglucocorticoid RU43044 (RU43) has a relative binding affinity for the GR that is comparable to Dex but is essentially devoid of any affinity for the PR or other steroid receptors (38). Because some of these compounds bind more weakly to the GR than Dex, a range of concentrations from 10^{-8} to 10^{-6} M was used for these studies. UL3 and A1-2 cells were treated with Dex, RU486, Org, ZK98, or RU43. Dex enhanced MMTV-Luc transcription 300–325-fold in UL3 cells (Fig. 1A). Treatment with Org or ZK98 induced at most a 3-fold activation of transcription at the highest concentration. In contrast, RU486 significantly induced transcription 60–75-fold depending on the concentration. RU43 at 10^{-6} M induced MMTV-Luc transcription 30-fold. Consequently, the antiglucocorticoids RU486 and RU43 exhibited significant agonist activity in the UL3 osteosarcoma cells.

The partial agonist activities of steroid receptor antagonists such as the antiestrogen, tamoxifen, and RU486 have been shown to depend on tissue and cell type. Thus, we compared the ability of these antiglucocorticoids to activate transcription in A1-2 breast cancer cell line (Fig. 1B). In A1-2 cells, Dex-induced MMTV-Luc transcription 1500–1750-fold depending on the concentration of hormone. In contrast to what was seen in UL3 cells, none of the antagonists, RU486, RU43, Org, or ZK98, significantly induced transcription (Fig. 1B). Thus, RU486 and RU43 exhibit significant agonist activity at the MMTV promoter in osteosarcoma cells but not in human breast cancer cells.

Glucocorticoid and RU486 Induce Chromatin Remodeling in Osteosarcoma Cells—Activation of target gene transcription in response to steroid treatment is a multistep process that includes remodeling of chromatin structure and assembly of a transcription preinitiation complex (39). The MMPV promoter undergoes a structural transition upon glucocorticoid treatment such that the region encompassed by the second nucleosome, Nuc-B, becomes hypersensitive to restriction endonucleases (25). Given the differential ability of glucocorticoids and antiglucocorticoids to induce transcription from the MMTV-Luc reporter in UL3 and A1-2 cells, we examined the capacity of the GR to mediate chromatin remodeling at Nuc-B. We monitored the alteration in chromatin structure by examining the changes in cleavage by restriction enzyme SstI that cleaves within Nuc-B (see Fig. 2, schematic). As shown in Fig. 2A, Dex treatment in UL3 cells resulted in elevated cleavage by SstI compared with untreated cells (cf. lanes C and D). Given that RU486 exhibited agonist activity in the osteosarcoma cell lines, we examined the capability of this antiglucocorticoid to induce GR-mediated restriction enzyme hypersensitivity. Treatment of UL3 cells with RU486 resulted in an enhanced level of SstI cleavage (comparable to that induced by Dex) (lanes C, D, and RU). In contrast, treatment with the antagonist ZK98 that fails to activate transcription also failed to elevate cleavage by SstI in UL3 cells. Examination of A1-2 cells demonstrated that only Dex but not RU486 or ZK98 treatment resulted in enhanced cleavage by SstI (Fig. 2B). These data are consistent with the...
partial agonist activity of RU486 at the MMTV promoter in UL3 cells but not A1-2 cells.

**Glucocorticoid and RU486 Induction of Transcription Factor Binding in Osteosarcoma Cells—**Previous *in vivo* analysis of the MMTV promoter showed that chromatin remodeling is highly correlated with transcription (22, 40, 41). In addition, chromatin remodeling is concomitant with the loading of transcription factors onto the promoter and the initiation of transcription (22, 26). To ascertain if the differential capacity of RU486 to induce MMTV-Luc transcription in UL3 and A1-2 cells was reflected in differential loading of transcription factors onto the promoter, we investigated nuclear factor 1 (NF1) binding by an *in vivo* exonuclease III footprinting assay (Fig. 3).

The addition of Dex resulted in a clear induction of NF1 loading onto the promoter in both UL3 (Fig. 3, cf. lanes 1 and 2) and A1-2 cells (Fig. 3 cf. lanes 4 and 5). In contrast, treatment with RU486 induced NF1 binding to the promoter in UL3 cells but not A1-2 cells (Fig. 3 cf. lanes 1 with 3 and 4 with 6). Thus RU486 treatment results in a level of GR-mediated chromatin remodeling and NF1 loading that is comparable to that induced by dexamethasone. These data are consistent with the ability of RU486 to induce partial agonist activity in the osteosarcoma cells (Fig. 1).

**Receptor Coactivators with the GR—**The isolation of an extensive array of intermediary proteins that interact with steroid receptors to modulate receptor activities has been one of the most significant developments in the field of hormone-induced activated transcription (42–44). Steroid receptors have been shown to interact in a ligand-dependent manner with coactivator complexes including proteins such as SRC-1/NCoA1, TIF-1, RIP-140, ERAP-160, p/CIP, and CBP/p300 as well as many others (42–44). MMTV activation by the GR depends on chromatin remodeling of the promoter as an obligatory first step (4). In the next set of experiments we examined GR interaction with the hBRG1 chromatin remodeling complex as well as the coactivators SRC-1/NCoA1, p/CIP, and CBP in both UL3 and A1-2 cells (Fig. 4). We compared GR protein/protein interactions following Dex, RU486, and Org treatment to see if these interactions correlated with the capacity of the hormones to induce activation of transcription. In a coimmunoprecipitation experiment, we detected a hormone-dependent association of the GR with hBRG1 upon treatment of osteosarcoma cells with either Dex or RU486 (Fig. 4A, cf. lanes 1–3). This is consistent with the capability of both of these compounds to induce chromatin remodeling at Nuc-B and activate transcription. However, Org was unable to induce GR association with hBRG1 (Fig. 4A, cf. lanes 1 and 4), congruent with its inability to...
FIG. 4. RU486-dependent association of GR with hBRG1 but not coactivators in UL3 cells. A, immunoprecipitation with an anti-GR antibody and immunoblotting of GR and hBRG1. UL3 and A1-2 cells were untreated (C) (lane 1) or treated with either dexamethasone (D) ($10^{-8}$ M) (lane 2) or RU486 (RU) ($10^{-8}$ M) (lane 3) or Org ($10^{-8}$ M) (lane 4) for 1 h. Immunoprecipitation with no antibody (No Ab) (lane 5) as a negative control indicates that the interaction is specific. B, UL3 and A1-2 differ in expression levels of hBRG1-associated factors. Whole cell extracts were prepared from UL3 and A1-2 cells and subjected to SDS-PAGE and Western blotting with antibodies specific for hBRG1, BAF 250, BAF 170, BAF 155, BAF 60a, and the GR. C, immunoprecipitation with anti-GR antibody and immunoblotting of GR and SRC-1/ NCoA1. UL3 and A1-2 cells were treated as in A. D, immunoprecipitation with anti-GR antibody and immunoblotting of GR CBP and pCIP. UL3 and A1-2 cells were treated as in A.
activate MMTV-Luc transcription (Fig. 1A). In contrast, in A1-2 cells only the agonist Dex was able to induce GR coimmunoprecipitation of hBRG1 (Fig. 4A, cf. lanes 1 and 2). RU486 or Org treatment did not promote GR interaction with hBRG1 above control levels (Fig. 4A, cf. lanes 1 with 3 and 4). The absence of a hormone-dependent GR/hBRG1 interaction upon RU486 treatment correlates with the inability of RU486 to induce chromatin remodeling and transcription factor loading at the MMTV promoter in A1-2 cells (Figs. 2 and 3).

The ability of the GR in UL3 cells to associate with the hBRG1 complex in the presence of RU486 could result from differences in the receptor or the relative levels of hBRG1-associated factors (BAFs) (45). One would anticipate that RU486 would induce similar GR conformation in both cell types so we focused on potential differences in the hBRG1-associated factors between the cell types. In the next series of experiments we examined the relative levels of the hBRG1 complex in both A1-2 and UL3 cells by SDS-PAGE and Western blotting. Both cell types express comparable levels of hBRG1 and BAF60a, although the BAF 60a isoforms differ between the two cell types (Fig. 4B). However, examination of BAFs 250, 170, and 155 reveal that these proteins are present at significantly higher levels in UL3 cells. In addition BAF 170 appears to have an additional isoform in UL3 cells (Fig. 4B). The higher levels of these components may permit RU486 association with the GR that is also expressed at higher levels in UL3 cells.

We also examined the association of the coactivators NCoA1, CBP, and p/CIP with GR upon Dex, RU486, and Org treatment (Fig. 4, C and D). Consistent with published data, Dex induces a hormone-dependent association of the GR with NCoA1, CBP, and p/CIP in both UL3 and A1-2 cells (Fig. 4, B and C, cf. lanes 1 and 2) (4). However, RU486 and Org were unable to induce this association in either cell line (Fig. 4, B and C, cf. lanes 1, 3, and 4). Therefore, in UL3 cells treatment with RU486 induces association of the GR with only a subset of the modulator proteins compared with what is observed with Dex treatment. The observation that RU486 does not induce GR association with the coactivators NCoA1, CBP, and p/CIP may partially explain why RU486 induces MMTV-Luc transcription at a level only 20% of that induced by Dex. The fact that Org fails to induce GR association with either hBRG1 or this group of coactivators may explain its pure antagonist activity (Fig. 1). Thus, upon RU486 treatment there is differential association of the GR with hBRG1, but not CBP, SRC-1/NCoA1, or p/CIP, in UL3 and A1-2 cells. The specific association of the GR with hBRG1 upon RU486 treatment in osteosarcoma cells may partially explain the mixed agonist/antagonist activity of RU486 in these cells.

**DISCUSSION**

Steroid receptor antagonists have been invaluable tools in the dissection of the molecular mechanisms underlying steroid receptor activation of transcription (16). The majority of studies examining the mixed antagonist/agonist activity of antihormones have been done with antiestrogens and antiprogestins (12, 30, 31). In case of estrogen receptor antagonists, some of the compounds have partial agonist effects on the skeletal system and have been used as therapeutic agents in the treatment of bone loss (46). In contrast to the protective effects of estrogens on bone, long term use of glucocorticoids in vivo induces bone loss (47). A comparison between the effects of antiguocorticoids on gene regulation in breast cancer cells and bone-derived osteosarcoma cells would give us an insight on the differences in regulatory mechanisms in the two cell types.

In this study the antiguocorticoids RU486 and RU43044 exerted significant agonist activity and activated MMTV-Luc transcription in osteosarcoma cells but not human breast cancer cells. In mouse breast cancer cells, although the GR/type II antagonist has been shown to bind to DNA, it was unable to activate transcription (3). Similar results have been observed with the antiestrogen tamoxifen that exhibits agonist activity in a tissue-dependent manner (48, 49). More directly, we show that the RU486-bound GR can effectively induce the chromatin transition necessary to allow the binding of NF1 to activate the MMTV promoter in osteosarcoma cells. Consistent with the ability to induce chromatin remodeling at Nuc-B, we detected an association of the GR with the hBRG1 complex in the presence of either RU486 or Dex. Although the precise mechanism for this interaction in UL3 cells is unknown, we did detect substantially higher levels of BAF 250, 170, and 155 along with the elevated levels of the GR in the UL3 cells relative to A1-2 cells.

Although the chromatin transition observed in the presence of either Dex or RU486 was similar in UL3 cells, the resulting transcriptional activation induced by RU486 was at a reduced level in UL3 compared with that induced by Dex. One explanation for this substantially lower activation was provided by an examination of GR association with the coactivators SRC-1/NCoA1, CBP, and p/CIP. Treatment of osteosarcoma cells with Dex resulted in the coimmunoprecipitation of GR and these coactivators. In contrast, RU486 did not promote GR association with any of the coactivators examined. Similarly, in the breast cancer cells none of the antagonists promoted the association with SRC-1/NCoA1, CBP, or p/CIP. The capability of hBRG1, but not coactivators, to associate with the RU486-bound GR provides an explanation for the partial transcriptional activation observed with the antiglucocorticoids in UL3 cells.

Previous studies revealed a requirement for the hBRG1 chromatin remodeling complex for glucocorticoid-dependent activation of MMTV (4). Association of the GR with coactivators in the absence of hBRG1 complex failed to activate transcription from chromatin templates (4). The present studies demonstrate that in osteosarcoma cells RU486 induces GR association with hBRG1 to partially activate MMTV transcription. This is consistent with an important role for chromatin remodeling in the activation of transcription, suggesting that there may be an intrinsic transcriptional activation potential in the remodeling process independent of the coactivators (16). However, this activation by RU486 is only ~20% that seen with a true agonist, implying that additional coactivators make significant contributions to the fully active GR at genes assembled as chromatin. Given the extensive clinical use of hormone antagonist in the treatment of breast cancer and other endocrine diseases, the capacity to predict if an individual antagonist will block the activity of a specific receptor, in one cell context but not another, may be of significant clinical importance.

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