9-cis-Retinoic Acid Inhibits Androgen Receptor Activity through Activation of Retinoid X Receptor

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Although the retinoic X receptor (RXR) forms heterodimers with many members of the estrogen receptor subfamily, the interaction between RXR and the members of the glucocorticoid receptor subfamily remains unclear. Here we show that the RXR can form a heterodimer with the androgen receptor (AR) under in vitro and in vivo conditions. Functional analyses further demonstrated that the AR, in the presence or absence of androgen, can function as a repressor to suppress RXR target genes, thereby preventing the RXR binding to the RXR DNA response element. In contrast, RXR can function as a repressor to suppress AR target genes in the presence of 9-cis-retinoic acid, but unliganded RXR can function as a weak coactivator to moderately enhance AR transactivation. Together, these results not only reveal a unique interaction between members of the two nuclear receptor subfamilies, but also represent the first evidence showing a nuclear receptor (RXR) may function as either a repressor or a coactivator based on the ligand binding status. (Molecular Endocrinology 19: 1200–1212, 2005)

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Abbreviations: AF, Transactivation function; AR, androgen receptor; ARA, AR-associated coregulator; ARE, androgen response element; CAT, chloramphenicol acetyltransferase; 9cRA, 9-cis-retinoic acid; CRBP II, cellular retinol-binding protein II; DBD, DNA-binding domain; DHT, dihydrotestosterone; ER, estrogen receptor; FBS, fetal bovine serum; GST, glutathione-S-transferase; LBD, ligand-binding domain; Luc, luciferase; MMTV, mouse mammary tumor virus; NR, nuclear receptor; PSA, prostate-specific antigen; RXR, retinoid X receptor; RXRE, RXR-response element; SDS, sodium dodecyl sulfate; siRNA, short interfering RNA; SRC, steroid receptor coactivator; TBST, Tris-buffered saline-Tween 20.

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The retinoic X receptor (RXR) is the NR for the vitamin A metabolite, 9-cis-retinoic acid (9cRA) (27, 28), and is involved in many biological processes, including cell growth, differentiation, metabolism, morphogenesis, and homeostasis during embryonic development and postnatal life (29, 30). Once bound with 9cRA, RXRs can bind to cognate DNA regulatory elements and activate transcription as homodimers (31). In the absence of ligand, RXRs form stable tetramers or serve as obligatory partners with a large number of NRs (32, 33). Therefore, the intracellular state of RXR is a dynamic equilibrium between tetramers, heterodimers, or homodimers, the populations of which are dependent on the vitamin A moiety and homeostasis.

To date, the cross-talk between the androgen signaling pathway and retinoid signaling pathway involves RXR repression of AR-mediated transactivation. This is shown in Figure 1A, where PC-3 cells were cotransfected with 300 ng MMTV-ARE-Luc and 100 ng pSG5-AR (lanes 6–20) with increasing amounts of pCMX-RXRα (+, 100 ng; ++, 300 ng; +++, 600 ng) as indicated. The total plasmid amount was adjusted with pCMX or pSG5 parent vector to 1 μg for each 35-mm transfection using SuperFect. phRL-tk-Luc (30 ng) was cotransfected as the control for normalization. B, PC-3 cells were cotransfected with 1 μg pSG5-AR, 3 μg MMTV-ARE-Luc, 0.1 μg phRL-tk-Luc, and increasing amounts of pCMX-RXRα (+, +++, ++, +) using SuperFect for each 100-mm transfection for 24 h, and then treated with 10 nM DHT and/or 9cRA for 16–18 h incubation. Total protein was extracted, and Western blot was performed to examine the expression of RXR and AR. C, PC-3 cells were cotransfected with 300 ng p(ARE)4-Luc and 100 ng pSG5-AR (lanes 6–20) with increasing amounts of pCMX-RXRα (+, 100 ng; ++, 300 ng; +++, 600 ng) as indicated. After 24 h transfection, cells were treated with DHT, 9cRA, and/or LG101305 as indicated. After 16–18 h incubation, cells were harvested for the Dual-Luciferase reporter assay. Each Luc activity is presented relative to the transactivation observed in the absence of DHT and is the mean ± SD of four experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (Figure continues on next page.)
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mains unclear. Here we identified the RXR as a novel coregulator for the AR by showing the interaction between the AR and RXR and their mutual regulation. The cross-talk between the AR and RXR not only expands the functions of both receptors but also contributes to the understanding of the complex gene network regulated by the NR superfamily.

RESULTS

9cRA Represses AR Target Genes Both in Vitro and in Vivo

Like the RXR, the AR acts as a transcription factor to activate many androgen target genes. To explore the cross-talk between the retinoid signaling pathway and the androgen signaling pathway, we investigated the potential regulatory effects of the RXR on AR-mediated transactivation. In PC-3 cells, the AR activated mouse mammary tumor virus (MMTV)-ARE-Luc activity in the presence of dihydrotestosterone (DHT) (Fig. 1A, lanes 6 and 9), which could then be further induced by the addition of the unliganded RXRα (lanes 18, 19, and 20 vs. lane 9). In contrast, without AR, RXRα by itself has no effect on the MMTV-ARE-Luc activity in the absence or presence of RXR ligands (lanes 2, 3, 4, and 5). However, in the presence of 9cRA, RXRα significantly suppressed DHT-induced AR transactivation in a dose-dependent manner (lanes 11, 12, and 13 vs. lane 9) but did not influence the AR expression (Fig. 1B). Furthermore, addition of higher concentrations of 9cRA has stronger suppressive effect than a lower concentration (10^{-6}, 10^{-7}, and 10^{-8} M 9cRA vs. 10^{-9} and 10^{-10} M 9cRA). An RXR-selective ligand, LG101305, was also used to examine the effect of activated RXRα on AR-mediated transcriptional activity. In the presence of LG101305, DHT-induced AR transactivation was also repressed by the RXRα in a dose-dependent manner (lanes 15, 16, and 17 vs. lane 9), although LG101305 was shown less potent than 9cRA (lanes 15, 16, and 17 vs. lanes 11, 12, and 13). Similar suppression effects also occurred when we replaced the MMTV-ARE-Luc reporter with the p(ARE)4-Luc reporter (Fig. 1C) or with the prostate-specific antigen (PSA)-Luc reporter (Fig. 1D), another AR target gene that is widely used as a marker for prostate cancer progression. To rule out the potential artificial effects linked to transfected reporter assays, the expression of endogenous PSA in LNCaP cells was measured by Northern and Western blot analyses. As shown in Fig. 2A, the expression of the PSA mRNA was dramatically induced after 24 h of DHT treatment. Addition of 1 μM 9cRA can clearly repress the expression of endogenous PSA mRNA in the presence of 10 nM DHT. The level of intracellular PSA protein detected by Western blotting was also reduced after the treatment of 1 μM or 1 nM RXR ligands (Fig. 2B). In addition, we also observed that in LNCaP cells transfected with PSA-Luc reporter, endogenous RXRα repressed DHT-
induced AR transactivation in the presence of 9cRA or LG101305 (Fig. 2C). This *in vivo* 9cRA-mediated suppressive effect strongly supports the reporter assay data in Fig. 1 and demonstrates that the RXR may function as a repressor in the presence of 9cRA to suppress AR target gene expression. In contrast, unliganded RXR can function as a weak coactivator to slightly enhance AR transactivation.

The AR Represses RXRα-Mediated Transactivation

The effect of the AR on RXRα-mediated transcriptional activity was also examined by transactivation reporter assay. The full-length AR and RXRα in eukaryotic expression vectors (pSG5-AR and pCMX-RXRα) were co-

transfected with a Luc reporter containing a RXR-response element (pCRBP II-Luc) into PC-3 cells. As shown in Fig. 3A, in the presence of 9cRA, the Luc activity induced by pCMX-RXRα could be repressed in a dose-dependent manner by cotransfection of pSG5-AR in the absence of DHT (lanes 6, 7, and 8 vs. lane 5), although addition of 10 nM DHT moderately reduced such repression (lanes 10, 11, 12 vs. 6, 7, 8). However, as shown in Fig. 3B, the increasing AR did not affect the protein expression level of RXRα. Using another strategy of cotransfection of an AR short interfering RNA (siRNA) plasmid into PC-3 cells, we found that AR siRNA, but not BS/U6, vector rescues the suppression effect of 9cRA-induced RXRα transactivation by the AR (Fig. 3C, lanes 4, 5, and 6 vs. lane 3), in a dose-dependent manner.

**Fig. 3.** The Effects of the AR on RXRα-Mediated Transcriptional Activity

A, PC-3 cells were cotransfected with 100 ng pCMX-RXRα (lanes 4–12), 300 ng pCRBP II-Luc, 30 ng phRL-tk-Luc, and increasing amounts of pSG5-AR expression vector (+, 100 ng; ++, 300 ng; ++++, 600 ng) in the presence (+) or absence (−) of 10 nM DHT and 1 μM 9cRA. The total plasmid amount was adjusted with pCMX or pSG5 parent vector to 1 μg for each 35-mm transfection using SuperFect. B, PC-3 cells were cotransfected with 1 μg pCMX-RXRα, 3 μg pCRBP II-Luc, 0.1 μg phRL-tk-Luc, and increasing amounts of pSG5-AR expression vector (+, 1 μg; ++, 3 μg; ++++, 6 μg) using SuperFect for each 100-mm transfection for 24 h, and treated with 10 nm DHT and/or 1 μM 9cRA for 16–18 h. Total protein was extracted, and Western blot was performed to examine the expression of RXR and AR. C, PC-3 cells were cotransfected with 60 ng pCRBP II-Luc and 15 ng pCMX-RXRα in the presence or absence of 90 ng pSG5-AR and increasing amounts of AR siRNA or BS/U6 empty vector (+, 90 ng; ++, 450 ng; ++++, 900 ng). pRL-TK (15 ng) was cotransfected as the control for normalization. The total plasmid amount was adjusted with pCMX, pSG5, or BS/U6 parent vector to 1 μg for each 35-mm transfection using SuperFect. After 24 h of transfection, cells were treated with or without 1 μM 9cRA. After 16–18 h incubation, cells were harvested for the reporter assay. Each Luc activity is presented relative to the transactivation observed in the absence of 9cRA and is the mean ± SD of four experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
RXRα Binding to the RXR-Response Element (RXRE) Is Inhibited by the AR

The EMSA using 32P-end-labeled RXRE-direct repeat 1 (DR1) probe (25) was applied to further dissect the mechanism of how the AR represses RXRα-mediated transactivation. As shown in Fig. 4, AR could not bind to DR1 (lanes 2 and 3) and the specific RXR-DR1 band was decreased with the addition of increasing amounts of the AR (lanes 5, 6, and 7 vs. lane 4). This result suggests that the AR might be able to repress RXRα-mediated transactivation by sequestering RXRα away from its target DNA.

AR-RXR Interaction in the Glutathione-S-Transferase (GST) Pull-Down Assays

The potential direct interaction between the RXR and the AR was then examined by the GST pull-down assay. The GST pull-down assay using GST-RXR fusion protein demonstrated that RXRα could physically interact with the AR (Fig. 5A). To map the regions in the AR that interact with the RXRα, various AR deletion mutants were tested in the GST pull-down assay. As shown in Fig. 5A, GST-RXRα can interact with the retinoic acid receptor-β and an in vitro-translated 35S-labeled AR deletion construct, the LBD of AR (AR-LBD), but not the N-terminal domain of AR (AR-N), the DBD of AR (AR-DBD), or the ERα. These results indicate that the RXRα can specifically interact with the AR-LBD. We also identified the regions in the RXRα that can interact with the AR. The N-terminal domain and the C-terminal domain of RXRα fused with GST (GST-RXRα-N+DBD and GST-RXRα-LBD, respectively) were used in the GST pull-down assay. As shown in Fig. 5B, in vitro-translated 35S-labeled AR can interact with both GST-RXRα-N+DBD and GST-RXRα-LBD. The minimal interaction regions in the N-terminal domain and the C-terminal domain of RXRα were defined by testing more RXRα fragments in the GST pull-down assay. As shown in Fig. 5C, the AR-LBD interacted with GST-RXRα-N and GST-RXRαH1–6, but not GST-RXRα-DBD, GST-RXRαH1–9, GST-RXRαH1–10, and GST-RXRαH11–12, suggesting that the N-terminal domain and helix 4/5/6 region of the RXR could bind to the LBD of the AR. The region in the AR-LBD domain required for interaction with RXRα was defined further by construction and testing of additional AR deletion derivatives in the GST pull-down assay. With serial deletion of AR-LBD starting from the C terminus, we created several truncated AR mutants. As shown in Fig. 6A, GST-RXRα had stronger interaction with both full-length AR and AR3H1–12 than with ARΔH6–12, ARΔH1–12, ARΔH3–12, and ARΔH1–12, and AR-N. Interaction with RXRα is not affected by deletion of helix 8 to helix 12 of AR but is affected by deletion of helix 6 to helix 12 of AR. These results indicate that helix 6/7 region of AR may play an important role in the AR-RXRα interaction. Based on the crystal structure of the human AR LBD (amino acids 663–919) in complex with the ligand metribolone (R1881) (35), helices 6 and 7 are shown to be exposed on the surface of AR-LBD by using 3D-Mol Viewer analysis (InforMax, Bethesda, MD) (Fig. 6B). The GST pull-down data combined with the structural analysis result imply that the helix 6/7 region of AR may serve as the interaction interface for the AR binding to the RXRα.

Coimmunoprecipitation of the RXR-AR Complex

We then used a coimmunoprecipitation assay to demonstrate that the AR could interact with the RXR in vivo. As shown in Fig. 7, A and B, the AR was detected in the anti-RXRα or anti-RXRβ antibody-precipitated complex from COS-1 cells cotransfected with the AR and the RXRα or the RXRβ, but not in the complex from cells transfected only with the AR, the RXRα, or the RXRβ. To further test whether the AR-RXR complex exists under physiological conditions, we used coimmunoprecipitation to detect an endogenous AR-RXR complex in cell lysates. As shown in Fig. 7C, the AR was detected in the anti-RXRα antibody-precipitated complex from LNCaP and PC-3(AR)2, but not PC-3 whole-cell extracts. Together, data from GST pull-down (Fig. 5, A and B, and Fig. 6A), and coimmunoprecipitation assays (Fig. 7) all demonstrated that the RXR interacts with the AR in vitro and in vivo.
Three AREs have been identified in the PSA gene. ARE I and ARE II reside in the -630 bp promoter region, and ARE III is in the enhancer region located -4 kb upstream of the PSA transcription initiation site (36). To examine whether the RXR can form a protein complex with the AR, which binds to an ARE in the promoter region of PSA gene, chromatin immunoprecipitation assays were performed to detect the recruitment of RXRα to the ARE I and ARE III regions.
after treatment with DHT. LNCaP cells were cultured in RPMI 1640 with 10% charcoal-treated fetal bovine serum (FBS) for 4 d followed by treatment with 10 nM DHT, 1 μM 9cRA, and/or 1 μM LG101305 for 3 h. Soluble chromatin was prepared after formaldehyde treatment of the cell cultures. Specific RXR antibodies were used to immunoprecipitate protein-bound chromatin fragments. The chromatin DNAs were analyzed by quantitative PCR using two specific pairs of primers spanning the ARE I region (39 to −250 bp) and the ARE III region (−4170 to −3978 bp) in the promoter of PSA gene. As shown in Fig. 8A, RXRα recruitment to ARE I was detected in LNCaP cells after treatment with DHT, but not 9cRA or LG101305, suggesting that the unliganded RXRα binds to the DHT-AR-ARE complex, which leads to the modest induction of AR transactivation (see Fig. 1A, lanes 18, 19, 20 vs. lane 9). However, in the presence of 9cRA or LG101305, AR recruitment to ARE I was significantly decreased in LNCaP cells with DHT treatment, which may result in the suppression of AR transactivation (see Fig. 1A, lanes 11, 12, 13 vs. lane 9, and lanes 15, 16, 17 vs. lane 9). Also, with DHT treatment, AR recruitment to ARE I was reduced in LNCaP cells in the presence of 9cRA or LG101305. No recruitment was detected to the promoter of the β-actin gene (data not shown). Similar results were also observed with
the ARE III region in the promoter of PSA gene (Fig. 8B). As the coimmunoprecipitation results showing that the interactions between the AR and the RXR were not influenced by DHT and/or 9cRA treatment, these chromatin immunoprecipitation assay data indicate that the activated RXR bound to 9cRA or LG101305 may have little effect on the interaction between AR and RXR, yet may play a major role in reducing the interaction between the AR and the ARE, leading to the repression of AR transactivation.

DISCUSSION

We have demonstrated a direct protein-protein interaction between the RXRα and AR and their mutual regulation of transcriptional activity. Figure 9A shows a model for AR suppressed RXR-mediated transactivation, with the 9cRA-inducible RXRE segment representing the entire RXR target gene promoter. In the presence of 9cRA, the RXR can form a homodimer and induce RXR-target gene transactivation through RXR homodimer binding to the RXRE. After AR binding to the RXR, the AR/RXR complex may dissociate from the RXRE, resulting in the repression of RXR target gene expression. However, because unliganded-AR, which is mainly located in the cytoplasm, has a stronger suppressive effect than liganded AR, it is also possible that the unliganded AR may bind to newly synthesized RXR in the cytoplasm and interfere with its nuclear translocation. On the other hand, Fig. 9B shows a model for the RXR modulation of AR-mediated transactivation. Without 9cRA binding, the RXR may stabilize the DHT-AR-ARE complex and enhance AR transactivation. In the presence of 9cRA, however, the AR-RXR complex may dissociate from the ARE, resulting in the suppression of AR transactivation.

It is also possible that mutual suppression between the AR and the RXR is due to competitive squelching of common limiting transcriptional factors. However, this is unlikely because our findings show that DHT-AR has a less suppressive effect on 9cRA-induced RXR transactivation than unliganded-AR. In contrast, only liganded-RXR represses AR-mediated transactivation.

The potential impact of this model may be significant. First, in contrast to the classic roles of RXR as a transcriptional coactivator to enhance the transactivation of its partner receptors, such as the vitamin D receptor, thyroid hormone receptor, and peroxisome proliferator activated receptor (37–40), we show here that RXR can suppress the transactivation of the AR, a member of another NR subfamily.

Second, because the presence or the absence of 9cRA binding can influence whether RXR functions as a repressor or weak coactivator for AR transactivation of reporter gene expression, our studies suggest that both ligand and receptor play important roles in the diverse functions of RXR. Similarly, the demonstration that DHT, a potent ligand for the AR, can reduce AR suppression of RXR transactivation also emphasizes that both ligand (DHT) and receptor (AR) may be necessary for proper AR function.

Third, because most evidence suggested that the AR functions as a transcription factor to activate its target genes via binding to AREs, data shown here demonstrate that the AR could also function as a repressor via protein-protein interaction to influence the expression of non-AR target genes, which represents a new dominant function for AR.

Fourth, both RXR and AR are widely expressed in various human tissues (4, 41–44), and cellular levels of RXR are generally substantially higher than AR levels. Early reports also established an inhibitory role of 9cRA in prostate carcinogenesis (45). The detailed mechanism by which 9cRA can suppress prostate tumor growth, however, remains unclear. Here we provide possible explanations by demonstrating that 9cRA may go through RXR-AR interaction to suppress PSA expression. Whether this newly discovered pathway can be used as a target to develop new drug(s) for the battle against prostate cancer remains to be elu-
cidated. Nevertheless, our conclusion that the AR and RXR modulate regulation of each other’s target genes is in agreement with a recent animal study showing that androgen negatively regulates RXR-mediated gene expression (46).

In summary, our demonstration of the AR-RXR interaction may represent a unique mechanism among NRs, and the transcriptional regulation between two receptors may help us to better understand the cross-talk between androgen and retinoid signaling pathways.

MATERIALS AND METHODS

Chemicals and Plasmids

DHT and 9cRA were obtained from Sigma Chemical Co. (St. Louis, MO). LG101305 was kindly provided by Ligand Pharmaceuticals, Inc. (San Diego, CA). pSG5-AR, MMTV-ARE-Luc, PSA-Luc, and pCMX-RXR were used in our previous reports (25, 34). pIRES-flag-AR was constructed by inserting flag-tagged AR cDNA into pIRESneo vector (CLONTECH, Palo Alto, CA). pCDNA3-RXR was from Renata Polakowska. p(ARE)4-Luc was kindly provided by Michael L. Lu (Harvard University, Boston, MA) (47). The AR siRNA expression vector that produces a siRNA targeting AR in mammalian cells was constructed by digesting and inserting a double-stranded polynucleotide into the Apal-EcoRI site of a DNA-based BS/U6 vector (Ambion, Inc., Austin, TX). pCRBPII-Luc was a gift from Vimla Band (Tufts University, Boston, MA).

Construction of Deletion Derivatives of AR and RXRα

To construct pCDNA3-flag AR (1–919), ARΔH1–12 (1–800), ARΔH6–12 (1–771), ARΔH4–12 (1–729), ARΔH3–12 (696), ARΔH1–12 (666), and AR-N (1–556), PCR amplification was used to generate the indicated fragment from the pSG5-AR with specific oligonucleotides that introduced in-frame BamHI and XbaI sites. PCR fragments were subcloned into pCDNA3-FLAG vector, a flag-tag insertion derivative of pCDNA3 vector (Invitrogen, San Diego, CA). To construct GST-RXRα-N (114–202), GST-RXRα-DBD (114–202), GST-RXRαH1–3 (203–265), GST-RXRαH6–9 (266–338), GST-RXRαH7–9 (339–409), and GST-RXRαH10–12 fusion proteins and GST control protein were obtained by transforming expressing plasmids into BL21 (DE3) pLysS strain-competent cells followed with 1 mM isopropyl-D-thiogalactoside induction.

Glutathione-S-transferase (GST) Pull-Down Assay

GST-RXRα, GST-RXRα-N, GST-RXRα-DBD, GST-RXRαH1–3, GST-RXRαH6–9, GST-RXRαH7–9, and GST-RXRαH10–12 fusion proteins and GST control protein were obtained by transforming expressing plasmids into BL21 (DE3) pLysS strain-competent cells followed with 1 mM isopropyl-D-thiogalactoside induction. GST fusion proteins were then purified by glutathione-Sepharose 4B as instructed by the manufacturer (Amersham Biosciences Corp., Piscataway, NJ). In vitro translated [35S]methionine-labeled proteins (5 μl) generated with the TNT-coupled reticulocyte lysate system (Promega Corp., Madison, WI) were mixed with the glutathione-Sepharose bound GST proteins at 4°C for 3 h to perform the pull-down assay as described previously (48). The bound proteins were separated on a 10% or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and visualized by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Cell Culture and Transfections

COS-1 cells and human prostate cancer PC-3 cells were maintained in DMEM containing penicillin (25 U/ml), streptomycin (25 μg/ml), and 10% FBS. Human prostate cancer LNCaP cells were maintained in RPMI 1640 with 10% charcoal-treated FBS. Briefly, 2 × 10^5 cells were plated on 35-mm dishes, 4 × 10^5 cells were plated on 60-mm dishes, and 10^5 cells were plated on 100-mm dishes 24 h before transfection. The cells were transfected using SuperFect

![Fig. 9. Schematic Models of Functional Interaction between AR and RXR](downloaded from mend.endojournals.org on September 2, 2005)
EMSA

The EMSA was performed as described previously (37). Briefly, the reaction was performed by incubating the 32P-end labeled DR1 probe with *in vitro*-translated RXRα with or without an increasing amount of the AR (1, 2, or 4 μg). The EMSA incubation buffer used included 10 mM HEPES/pH 7.9, 2% (vol/vol) glycerol, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, and 1 mM dithiothreitol. DNA-protein complexes were resolved on a 5% native polyacrylamide gel. The radioactive gel was analyzed by PhosphorImager scanning (Molecular Dynamics).

Western Blotting

Cell lysates or immunoprecipitated proteins were separated by SDS-PAGE through 10% gels, electroblotted onto polyvinylidene difluoride membrane, and Western blotted with the indicated antibodies. Blots were blocked at 4°C in Tris-buffered saline with 0.5% Tween 20 (TBST) prior to incubation with the appropriate primary antibodies. After incubation, the blots were washed in TBST and then incubated with the appropriate alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology, Inc). Washed blots were developed with [1:1000] or rabbit anti-AR (1:3000) (NH-27) antibodies.

Chromatin Immunoprecipitation

LNCaP cells were cultured in RPMI 1640 with 10% charcoal-treated FBS for 4 d, treated with appropriate ligands for 3 h, washed with PBS, and cross-linked with 1% formaldehyde at 37°C for 10 min. Then the cross-linking was stopped by adding glycine to a final concentration of 125 mM for 5 min at room temperature. Cells were rinsed twice with ice-cold PBS, collected into PBS, and centrifuged. The pellets were then resuspended in cell lysis buffer [5 mM NaHPO4/pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.5% (wt/vol) Nonidet P-40, 0.1% (wt/vol) SDS, 1% (wt/vol) sodium deoxycholate, and 1 mM phenylmethylsulfonylfluoride]. Insoluble material was removed by centrifugation (16,000 g; 10 min at 4°C). Polyclonal anti-RXRα or anti-RXRβ antibodies (10 μg) (200 μg/ml; Santa Cruz Biotechnology, Inc.) were added to the cell lysates and incubated for 2 h at 4°C. Immunoprecipitates were collected with protein A/G-Sepharose beads (Santa Cruz Biotechnology, Inc.) washed four times in RIPA buffer, and then analyzed by Western blotting with mouse anti-flag (1:1000) or rabbit anti-AR (1:3000) (NH-27) antibodies.
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