The Androgen Receptor Recruits Nuclear Receptor CoRepressor (N-CoR) in the Presence of Mifepristone via Its N and C Termini Revealing a Novel Molecular Mechanism for Androgen Receptor Antagonists*

Received for publication, August 5, 2004, and in revised form, November 23, 2004
Published, JBC Papers in Press, December 14, 2004, DOI 10.1074/jbc.M408972200

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The androgen receptor (AR) activates target gene expression in the presence of agonist ligands via the recruitment of transcriptional coactivators, but recent work shows that overexpression of the nuclear corepressors NCoR and SMRT attenuates this agonist-mediated AR activation. Here we demonstrate using NCoR siRNA and chromatin immunoprecipitation that endogenous NCoR is recruited to and represses the dihydrotestosterone (DHT)-liganded AR. Furthermore this study shows that NCoR and coactivators compete for AR in the presence of DHT. AR antagonists such as bicalutamide that are currently in use for prostate cancer treatment can also mediate NCoR recruitment, but mifepristone (RU486) at nanomolar concentrations is unique in its ability to markedly enhance the AR-NCoR interaction. The RU486-ligated AR interacted with a C-terminal fragment of NCoR, and this interaction was mediated by the two most C-terminal nuclear receptor interacting domains (RIDs) present in NCoR. Significantly, in addition to the AR ligand binding domain, the AR N terminus was also required for this interaction. Mutagenesis studies demonstrate that the N-terminal surface of the AR-mediating NCoR recruitment was distinct from tau5 and from the FXXLF motif that mediates agonist-induced N-C-terminal interaction. Taken together these data demonstrate that NCoR is a physiological regulator of the AR and reveal a new mechanism for AR antagonism that may be exploited for the development of more potent AR antagonists.

The androgen receptor (AR), a member of the steroid/nuclear receptor superfamily, plays a critical role in normal male development, including the development of the prostate gland. In addition, AR action plays a fundamental role in the development and progression of prostate cancer (1–3). Prostate cancers are initially androgen responsive such that targeted therapies aimed at lowering circulating androgen levels are the treatment of choice for metastatic disease. In most cases, however, the disease becomes progressive and unresponsive to androgen ablation therapies. This progressive stage of the disease, referred to as hormone refractory or androgen-independent prostate cancer, is generally heralded by the re-expression of androgen-regulated genes such as prostate-specific antigen (PSA). AR gene amplification or mutations may contribute to this re-expression of androgen-regulated genes, but it occurs mainly through undefined molecular mechanisms that allow for AR signaling in the absence of ligand or at reduced systemic androgen levels (4–8). Thus, new approaches to silence AR signaling may have important therapeutic ramifications for the therapy of both early and late stage prostate cancer.

Members of the steroid receptor superfamily signal in a similar fashion based on their structural similarity (9). The addition of an agonist ligand leads initially to the DNA binding of the receptor, followed by the ordered recruitment of both transcriptional coactivators and other mediators to the ligand binding domain. This leads to histone modifications including acetylation, and finally to transcriptional activation (10). In contrast, in the presence of a hormone antagonist, steroid receptors fail to recruit coactivators. Moreover, certain antagonists preferentially stimulate the recruitment of nuclear receptor corepressors to target promoters, which in turn recruit a multiprotein complex that leads to histone deacetylation and transcriptional repression (11–14).

The AR is structurally similar to other steroid receptors in that it is recruited to target elements as a homodimer and contains a high affinity, steroid-specific ligand binding domain. However the AR differs in the molecular mechanisms by which it recruits both coactivators and corepressors. Unlike other family members, the ligand-binding domain of the AR preferentially recruits its own AF-1 domain via an N-terminal FXXLF motif, which interacts strongly with the hydrophobic cleft of the LBD created by ligand binding (15–19). The subsequent recruitment of coactivators, in particular the SRC family, is then mediated primarily by the AF-1 domain rather than by the LBD (18, 20–23). In addition, we and others (24–28) have shown that the agonist-ligated AR can recruit the corepressors NCoR and SMRT, suggesting that agonist-dependent activation of the AR may be dependent upon the relative levels of these corepressors versus coactivators.

*This work was supported by Grants DK61047 (to S. P. B.) and DK56123 (to A. N. H.) from the National Institutes of Health, Department of Defense Grant PC040246, the Dana Farber Harvard Cancer Center Prostate SPORE, and the Hershey Family Prostate Cancer Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; PSA, prostate-specific antigen; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; LBD, ligand binding domain; RLU, relative light units; RID, receptor interacting domains; HA, hemagglutinin; DBD, DNA binding domain; CMV, cytomegalovirus; NCoR, nuclear corepressor; siRNA, small interfering RNAs.
Besides the physiological agonists testosterone and DHT, the AR can also interact with many other steroidal or nonsteroidal drugs that function as relatively pure antagonists (such as hydroxyflutamide and bicalutamide) or as partial agonists (29). Bicalutamide, which is widely used for prostate cancer treatment, can stimulate the AR to bind DNA, but fails to recruit coactivators and can mediate the recruitment of NCoR to the androgen-regulated PSA gene, indicating that corepressor recruitment may contribute to antagonist activity (30–32). Nonetheless, bicalutamide has limited efficacy in the advanced androgen-independent stage of prostate cancer, and other AR antagonists are similarly ineffective at this stage of the disease (33). As enhancement of corepressor recruitment to the AR may be an effective approach for blocking AR signaling in prostate cancer, this study further examines corepressor recruitment by AR agonists and antagonists.

Loss of function experiments were carried out initially using NCoR siRNA, in conjunction with chromatin immunoprecipitation (ChIP). These confirmed that endogenous NCoR could negatively regulate the activity of the DHT-liganded AR, and indicated that AR activity may be regulated by the relative levels of NCoR and coactivators. Although multiple other ligands could mediate AR-NCoR interaction, the AR partial agonist RU486 (mifepristone) functioned uniquely, at nanomolar concentrations, as a strong enhancer of this interaction. Significantly, while NCoR RIDs and the AR LBD contributed to AR-NCoR binding, this was markedly enhanced by a further interaction with the AR N terminus via a site that was independent of the N-terminal FXLLF motif. These results demonstrate that NCoR is a biological regulator of AR action, and identify a new role for the AR N terminus in the AR-NCoR interaction. Moreover, the marked enhancement of the AR-NCoR interaction by RU486, but not by AR antagonists currently in clinical use, indicates that this interaction is a target for the development of new potent AR antagonists.

MATERIALS AND METHODS

Plasmids and Reagents—Expression vectors for AR (p5VAro) Era (pcDNA-ERAo), NCoR (PKCR2-NCoR), and SRC1 (pS6S-SRC1) have been described previously (24, 34, 35). The NCoR vector (pPKCR2-NCoRc) was referred to previously as NCoR and encodes the C-terminal amino acids 1574–2454 of NCoR (numbering is based on murine NCoR). VP16-NCoR encodes the three NCoR RIDs from NCoR fused to the VP16 transactivation domain in the AASVP16 vector (amino acids 661–919). The additional fusion protein was constructed in the pACT vector, while fusions to the Ga4 DNA binding domain were in the pBind vector (Promega, Madison, WI). The VP16 fusions with AR include VP16-AR (encoding the full-length AR), VP16-ARDBD-LBD (encoding amino acids 501–919), VP16-ARNTD (amino acids 1–500), VP16-A5LBD (amino acids 661–919), and have been described (24). Additional NCoR fragments fused to VP16 were generated by ligating the indicated PCR-amplified regions (numbering is based on the murine NCoR sequence) into pACT with a C-terminal HA tag. GST-NCoR (N2N1) contains amino acids 2063–2300 of hNCoR fused to GST in PGEAT1 and has been described previously (36, 37). The AR mutant F23A was kindly provided by E. Wilson. The mutants LF26,27A and ARdel23–27 were generated from p5VAro using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Gal4-NCoR CoSRN box mutants were described previously (34, 35). The reporter constructs ARE5 Luciferase, containing four tandem copies of a synthetic ARE, and the estrogen response element (ERE)-luciferase containing two EREs have been described (24). pG5-luciferase, regulated by five tandem Gal4 binding sites, and pRL-CMV, a CMV luciferase containing two EREs have been described (24). Additional NCoR fragments fused to VP16 were generated in the pBind vector, while fusions to the Ga4 DNA binding domain were in the pACT vector, and coregulators (NCoR, NCoRc, SRC1) except where indicated, and with 1.25 ng of pRL-CMV Renilla vector for normalization. After 24 h the medium was replaced with fresh DMEM/10% CDS-FBS medium containing hormones or drugs at the indicated final concentrations. Following a further 24 h, firefly, and Renilla luciferase activities were assayed with the dual-luciferase assay system from Promega as per the supplier's instructions. All samples were in triplicate or quadruplicate and firefly luciferase activities were normalized for cotransfected Renilla activity. To assess expression of transfected proteins, lysates from replicate wells were prepared in 1% SDS, run on 8% or 10% SDS-PAGE, and immunoblotted with anti-HA (Covance) or anti-AR antibodies (Santa Cruz Biotechnology) (1:1000 dilutions incubated overnight at 4 °C), followed by horseradish peroxidase-conjugated anti-mouse or rabbit Ig secondary antibodies (Promega) and ECL (Amer sham Biosciences).

RNA Interference—Vectors expressing hairpin small interfering RNAs (siRNA) under the control of the mouse U6 promoter were constructed by inserting part of annealed DNA oligonucleotides into pBS/U6 plasmid (a gift of Y. Shi) at Apal-EcoRI sites (38). The target sequence for NCoR was 5′-GGGTTTATGAGACCACCTATGAG-3′. To assess effects on AR transactivation, this siRNA plasmid (pU6-NCoR) or a control lamin siRNA plasmid (pU6-laminin) were cotransfected with the AR expression vector and the indicated reporters. To assess protein expression, cells in 6-well plates were transfected with 0.1–1.6 μg of each reporter well. At 48 h after transfection, cell lysates and nuclear extracts were isolated (39). Nuclear proteins were separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred overnight to nitrocellulose membranes. Blots were probed with 1:500 dilution of an affinity-purified anti-NCoR antisera in Tris-buffered saline containing 5% nonfat milk and 0.05% Tween 20, followed by horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) at 1:10000 dilution (24). The blots were visualized using ECL Plus Western blotting detection system.

Chromatin Immunoprecipitation—LNCaP prostate cancer cells grown to ~80% confluence in 10-cm plates were switched to steroid hormone-depleted medium (RPMI 1640/10% CS-FCS) for 48 h and then exposed to 10 nM DHT for varying times. Plates were then rinsed with PBS and fixed for 10 min at room temperature with 1% formaldehyde in PBS. Cells were then washed twice with ice-cold PBS, crosslinked by scraping cells into 1 ml of 100 mM Tris, pH 9.4 and 10 mM dithiothreitol and incubating at 30 °C for 15 min. Cell pellets were then washed twice with PBS and resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and protease inhibitors). Chromatin was sheared to 300–1000 bp with three sequential 10 s pulses at 70% power with a microtip ultrasonic cell dismembrator (Fisher). Cell debris was removed at 10,000 × g and the supernatant was precleared with 10 μg of sheared salmon sperm DNA, 20 μl of non-immune serum, and 20 μl of a 50% slurry of protein A-Sepharose. Immunoprecipitation was with 500 ng of AR N-terminal rabbit polyclonal antibody (PG-21, Upstate Biotechnology), affinity-purified NCoR C-terminal anti-peptide antibody (24) or a nonspecific control antibody, 2 μg of an additional salmon IgG control, and 20 μl of a 50% slurry of protein A-Sepharose. Precipitates were washed three times with 300 mM NaCl, 50 mM Tris, pH 8.0, 2.7 mM KCl, 0.05% Tween-20 and 1% deoxycholate. Three additional washes with 10 mM Tris, 1 mM EDTA were performed and then beads were eluted three times with 35 μl of 1% SDS and 100 mM NaHCO3, at 37 °C for 10 min each. Eluates were pooled and incubated at 65 °C overnight to reverse cross-links. Products were then purified with QiaQuick PCR purification spin resin (Qiagen, Valencia, CA) and 10% of the eluate was subjected to 50 cycles of PCR amplification with steps of 94 °C, 55 °C, and 72 °C for 1 min each. The primers for the p21 cyclin-dependent kinase promoter were 5′-AAGCTCCCCTCGAGACCCAGA-3′ and 5′-CAAGCCTGACAGACTGAC-3′; and for the PSA promoter 5′-GAGAGCTTACACCTGTTGCTT-3′ and 5′-AGTTCTGAATGTTGCTTCA-3′. PCR products were analyzed by gel electrophoresis and ethidium bromide staining in 4% agarose gels.

**GST Pull-down Assays—**GST-NCoR (N2N1) and GST alone were produced as previously described and purified using glutathione-agarose beads (36, 37) and there integrity was visualized on SDS-PAGE. To derive the indicated AR moieties, CV1 cells in 60-mm plates were transfected with either full-length AR, AR N-BDB, or D-LBD as described above and displaced with rifampicin or without 10 nM DHT or RU486. Cells were washed twice with ice-cold PBS and extracts prepared by scraping cells into 0.75 ml of lysis buffer (PBS, 5% glycerol, 0.05% Triton X-100 and protease inhibitors). Lysates were incubated on ice for 5 min and sonicated with two sequential 5 s pulses at 30% power with a microtip ultrasonic dismembrator (Fisher) and centrifuged at 4 °C to pellet insoluble material. Cell extracts were precleared with GST agarose beads for 2 h at 4 °C on a rotating wheel.
Cleared lysates were incubated for 16 h with or without 10 nM DHT or RU486 at 4 °C on a rotating wheel in the presence of equal amounts of GST or GST-NCoR (N2N1) agarose beads. The beads were washed once with ice-cold lysis buffer and three times with ice-cold PBS and bound proteins were eluted by boiling in Laemmli sample buffer (Promega), run on 12% SDS-PAGE and blotted onto nitrocellulose membranes. To visualize AR proteins, Western blots were probed with NCoR antiserum and exposed using chemiluminescence. NCoR immunoblotting of transferred proteins were probed with NCoR antiserum and exposed using chemiluminescence.

RESULTS

NCoR Down-regulation Enhances AR Transcriptional Activity—Previous studies have shown that transfected NCoR could repress the transcriptional activity of the DHT-ligated AR (24). RNAi was used to directly address whether endogenous NCoR functions as a negative regulator of AR transcriptional activity. CV1 cells were transfected with an AR expression vector with the LBD-deleted (AR N-DBD, encoding the N terminus and DBD, AR N-DBD) and AR full-length AR, an AR expression vector with the LBD-deleted (AR N-DBD, encoding the N terminus and DBD) was constitutively active in the absence of DHT and was not stimulated by the NCoR siRNA (Fig. 1C).

As a further control, we examined the estradiol-ligated ERs. Whereas NCoR can interact with ERs when it is liganded by certain partial agonists, the estradiol-ligated ERs is not repressed by NCoR transfection. As shown in Fig. 2, the NCoR siRNA did not enhance the activity of the estradiol-ligated ERs, while AR activity was again markedly enhanced in the same experiment. Taken together, these data supported the conclusion that endogenous NCoR functions to suppress the transcriptional activity of the agonist-ligated AR.

NCoR Is Recruited to Androgen-regulated Genes by the Endogenous DHT-ligated AR in Prostate Cancer Cells—Although we have been able to markedly down-regulate NCoR expression by siRNA and enhance AR activity in CV1 and 293T cells (data not shown) efforts to substantially down-regulate NCoR protein in a prostate cancer cell line expressing endogenous AR have not yet been successful. Therefore, chromatin immunoprecipitation was used as an alternative approach to test the hypothesis that NCoR associates with the DHT-ligated endogenous AR and is recruited to AR-regulated genes. Androgen responsive LNCaP prostate cancer cells were grown in steroid hormone-depleted medium for 2 days, and were then pulsed with DHT. Chromatin was cross-linked with formaldehyde at varying times after the DHT pulse, and sheared chromatin was immunoprecipitated with anti-AR, anti-NCoR, or control antibodies. PCR was then used to assess AR and NCoR recruitment to AREs in the p21 cyclin-dependent kinase inhibitor promoter and PSA regulatory regions. As shown in Fig. 3A, DHT treatment led to the recruitment of AR to the p21 gene. Significantly, NCoR was not associated with the p21 ARE in the absence of DHT, consistent with the lack of AR binding, but became associated after DHT treatment. Interestingly, the binding of both AR and NCoR appeared to be transient based on this method, as has been reported previously for AR and ERs (31).

DHT similarly induced the association of both AR and NCoR with the PSA promoter (Fig. 3B), as well as the ARE in the PSA enhancer (data not shown). In this experiment the AR and NCoR association was not detected until 30–45 min, and it persisted for at least 2 h. More rapid and transient association of both AR and NCoR have been observed in other experiments (data not shown), and the basis for the variable recruitment kinetics are not yet clear. Nonetheless, a consistent correlation has been observed between AR and NCoR recruitment in re-
response to DHT. It should be noted that previous studies have shown NCoR recruitment by antagonist (bicalutamide)-liganded AR, but not the DHT-ligated AR (31, 32). The basis for this difference is not clear, but may reflect the distinct affinity purified anti-NCoR antibodies used in this study. In any case, these data in conjunction with the siRNA results indicate that endogenous NCoR contributes to the regulation of AR transcriptional activity.

**NCoR Represses SRC-1 Enhancement of AR Transcriptional Activity**—We reported previously that NCoR repression of AR transcriptional activity was independent of the HDAC-interacting repressor domains in the NCoR N terminus, as repression was observed in the presence of trichostatin A (24). Moreover, AR could be repressed by the NCoR C terminus, encoding the receptor-interacting domains (RIDs), independently of the repressor domains in the NCoR N terminus. Significantly, repression was abrogated by mutations in the three NCoR CoRNR box motifs (IXXII) present in the RIDs, which are presumed to interact with helices 3–5 in the coactivator/corepressor binding site of the AR LBD. This suggested that repression might be caused by NCoR inhibition of the AR N-C-terminal interaction, as an LXXLL-like motif in the NCoR C terminus normally associates with the AR LBD and makes a major contribution to AR transcriptional activity. However, NCoR could also repress AR activation by partial agonists that do not mediate AR N-C-terminal interaction, and did not block the interaction between the AR N terminus and the DHT-ligated AR LBD in mammalian two-hybrid protein interaction assays (data not shown).

An alternative possible mechanism for AR repression by NCoR is inhibition of coactivator binding. Consistent with this mechanism, transfection of the NCoR C terminus strongly repressed AR coactivation by SRC-1 (Fig. 4A). Importantly, previous studies have shown that SRC-1 binding to the AR is mediated primarily by the AR N terminus, with little or no interaction between the NR boxes in SRC-1 and the AR LBD. This suggested that NCoR might be inhibiting SRC-1 binding by interacting directly with the AR N-terminal domain. This was tested by examining whether the NCoR C terminus, expressed at high levels, could repress the constitutive activity of the AR N-terminal domain.

CV1 cells were transfected with an AR N-DBD fragment (encoding the AR N terminus and DNA binding domain), which had high androgen-independent activity on an ARE reporter gene when fused to VP16 (Fig. 4B). Significantly, this activity was strongly repressed by cotransfection with NCoRc. It should be noted that this repression required the high level expression of transfected NCoRc, consistent with a relatively low affinity interaction and the failure of NCoR siRNA to enhance the activity of the AR N-DBD fragment (Fig. 1C). To confirm that this interaction was not dependent on the VP16 domain, we tested the AR N-DBD fragment alone on the ARE reporter. As shown in Fig. 4C, this construct had less activity than the VP16 fusion, but was also inhibited by cotransfected NCoRc. The repression seen in these experiments was specific as there was no effect on control CMV-regulated reporters (data not shown). Moreover, although NCoRc can interact with the AR LBD, it did not repress the transcriptional activity of the AR DBD and LBD fused to the VP16 transactivation domain (pACT-AR DBD-LBD) (Fig. 4D). Together, these results indicated that NCoR can interact with both the AR N and C termini, and that the relative levels of NCoR versus coactivator proteins may regulate AR transcriptional activity.

**NCoR Interaction with AR Is Enhanced by RU486**—To further assess the mechanisms mediating NCoR interaction with AR, and whether NCoR binding can be influenced by the conformation of the AR LBD, we next examined a series of AR partial agonists and antagonists. Of particular interest was whether enhanced NCoR binding might contribute to the effects of certain AR antagonists. To directly assay NCoR-AR binding, we carried out mammalian two-hybrid protein interaction assays using the NCoR C terminus fused to the Gal4 DBD (Gal4-NCoRc) and full-length AR fused to the VP16 translational activity of the AR N-DBD, we next examined a series of AR partial agonists and antagonists. Of particular interest was whether enhanced NCoR binding might contribute to the effects of certain AR antagonists. To directly assay NCoR-AR binding, we carried out mammalian two-hybrid protein interaction assays using the NCoR C terminus fused to the Gal4 DBD (Gal4-NCoRc) and full-length AR fused to the VP16 transactivation domain (pACT-AR DBD-LBD) (Fig. 4D). Together, these results indicated that NCoR can interact with both the AR N and C termini, and that the relative levels of NCoR versus coactivator proteins may regulate AR transcriptional activity.

**Androgen Receptor and Nuclear Corepressors**

![Androgen Receptor and Nuclear Corepressors](image-url)
activation domain (VP16-AR), in conjunction with the pG5-luciferase reporter (containing five tandem Gal4 elements recognized by the Gal4 DBD). Consistent with our previous data, a weak ligand-independent interaction between NCoR and AR could be detected, and this was not enhanced by DHT (Fig. 5A). Similarly to DHT, the NCoR-AR interaction was not substantially enhanced by a series of other partial agonists or antagonists, including bicalutamide (Fig. 5A), hydroxyflutamide, cyproterone acetate, estradiol, progesterone, or androstenedione (data not shown).

In contrast, the NCoR-AR interaction was markedly enhanced by RU486 (mifepristone) (Fig. 5A). RU486 was originally identified as a steroidal antagonist of the GR and PR, and more recent data have shown that NCoR interacts with the RU486-ligated GR and PR (13, 40–42). The unique structural feature of RU486 is a bulky 11β substitution that appears to interact with helix 3 and prevent the formation of the coactivator binding site. To confirm that RU486 could mediate NCoR recruitment to the unmodified AR, we examined the effect of a VP16-NCoRc fusion protein on AR transactivation of an ARE-regulated reporter gene. The VP16-NCoRc construct did not significantly enhance the transcriptional activity of the DHT-ligated AR, and was inhibitory in some experiments (presumably due to disruption of coactivator recruitment despite the VP16 transactivation domain) (Fig. 5B). In contrast, the RU486-ligated AR was markedly coactivated by VP16-NCoRc, indicating that NCoR could be strongly recruited to an ARE by the unmodified RU486-ligated AR.

Given that these experiments employed a multimerized artificial ARE, we next asked whether NCoR could be recruited by RU486 to the physiological AREs regulating the PSA gene. As shown in Fig. 5C, RU486 functioned as a weak partial AR agonist when assayed on a luciferase reporter regulated by the PSA upstream region, containing the androgen-regulated PSA promoter and enhancer. Cotransfection with VP16-NCoRc did not substantially alter DHT-stimulated activity, but markedly enhanced the activity of the RU486-ligated AR, confirming NCoR recruitment to a physiological reporter. These results suggested that recruitment of endogenous NCoR might contribute to the AR antagonist activity of RU486. This was tested by cotransfection with an NCoR siRNA construct. As shown in Fig. 5D, the weak agonist activity of RU486 toward the AR was enhanced by NCoR siRNA, indicating that recruitment of endogenous NCoR contributed to RU486 antagonist activity. It should be noted that the level of transcriptional activity obtained with the RU486-ligated AR and NCoR siRNA was still modest compared with the DHT-ligated AR. This modest activity is consistent with the minimal ability of the RU486-ligated AR to recruit coactivators, as assessed by SRC-1 or SRC-2 cotransfections (data not shown), and lack of N-C-terminal interaction (see below), but may also reflect recruitment of other corepressors.

**AR N Terminus Is Required for NCoR Recruitment by the RU486-ligated AR**—Further experiments were carried out to determine the mechanism of NCoR interaction with the RU486-ligated AR. Binding to the AR LBD was assessed using an AR DBD-LBD construct, which has minimal transcriptional activity in the absence of the N terminus on a ARE (ARE4-Luc-Fig. 6A). The AR N terminus (expressed as a VP16-AR N-terminal fusion protein) interacted with the AR DBD-LBD in the presence of DHT, reflecting the LXXLL-like motif in the AR N terminus that interacts strongly with the agonist-activated coactivator binding site in the AR LBD (Fig. 6A). In contrast, RU486 did not induce an interaction between the AR N terminus and LBD, consistent with the RU486-ligated AR LBD assuming a non-agonist conformation. However, despite the strong interaction between NCoR and the RU486-ligated full-length AR, the RU486-ligated AR DBD-LBD failed to interact detectably with the VP16-NCoRc protein (Fig. 6A).

Similar results were obtained in a two-hybrid protein binding assay using the AR LBD fused to the Gal4 DNA binding domain. This fusion protein interacted very strongly with the AR N terminus in the presence of DHT, but there was no detectable interaction between the RU486-ligated Gal4 AR-LBD and VP16-NCoRc (Fig. 6B). Efforts to detect such an interaction using higher concentrations of RU486 (up to 10 μM) or of the VP16-NCoRc protein were also unsuccessful (Fig. 6B).
samples. The data are expressed as RLU. Luciferase activities were determined from triplicate samples. The indicated ligands were added for 24 h, and luciferase activity with the pG5-Luc and pRL-CMV reporters in the presence of Gal4-NCoRc and AR DBD-LBD in the presence of ARE4-Luc and the pRL-CMV control. The indicated ligands were added for 24 h, and luciferase activity was determined from triplicate samples. The data are expressed as RLU. Luciferase activities were determined from triplicate samples. The indicated ligands were added for 24 h, and luciferase activity with the pG5-Luc and pRL-CMV reporters in the presence of Gal4-NCoRc and AR DBD-LBD in the presence of ARE4-Luc and the pRL-CMV control. The indicated ligands were added for 24 h, and luciferase activity was determined from triplicate samples. The data are expressed as RLU. Luciferase activities were determined from triplicate samples. The indicated ligands were added for 24 h, and luciferase activity was determined from triplicate samples. The data are expressed as RLU.

Finally, in the converse two-hybrid experiments we have not detected an interaction between the RU486-ligated VP16-AR LBD and Gal4-NCoRc, while the full-length RU486-ligated AR is strongly recruited by Gal4-NCoRc (Fig. 6A). Taken together these data indicated that the recruitment of NCoR to the RU486-ligated AR required both the AR N terminus and LBD.

NCoR Binding to the RU486-ligated AR Is Receptor Interaction Domain-specific—Although NCoR binding to the RU486-ligated AR LBD alone could not be detected in the above experiments, we hypothesized that one or more of the NCoR RIDs contributed to the strong interaction between NCoR and the RU486-ligated full-length AR. Therefore, a series of NCoR mutants that lacked individual RIDs and thus their respective CoRNR boxes were fused to the Gal4-DNA binding domain and tested for interaction with the full-length AR fused to the VP16 activation domain. As shown in Fig. 7, deletion of the N-terminal RID, N3 (Gal4-N2N1), which is required for NCoR to bind the TR, enhanced the interaction with the RU486-ligated AR, as well as with the DHT-ligated and -unliganded AR. This enhancement was not due to increased expression, as all of the constructs were expressed at similar levels (data not shown), and may reflect more optimal folding. Consistent with the lack of a role for N3, the Gal4-N3 construct was inactive. In contrast, deletion of the C-terminal N1 RID (Gal4-N3N2), abrogated recruitment of the RU486-ligated AR. Significantly, we have shown previously that this construct (Gal4-N3N2) is able to strongly recruit the unliganded TR (34).

To confirm and extend these findings in the context of an intact AR, we generated additional NCoR constructs fused to the VP16 activation domain. Consistent with the above results, the VP16-NCoR (2021) construct (deletion of N3) interacted very strongly with the RU486-ligated AR (Fig. 7B). The removal of N2 in the VP16-NCoR (2083) construct resulted in decreased activity. This was not because of lower expression as these constructs were similarly expressed (data not shown), and indicated that N2 contributed to the AR interaction. The deletion of N1 in the VP16-NCoR (2294) construct further reduced interaction with the RU486-ligated AR to baseline levels. Significantly, this latter VP16-NCoR (2294) construct still contains a putative C-terminal LXXLL NR box shown previously to interact with the ER, indicating that this NR box does not mediate the AR interaction (44). Taken together these data indicate that both N2 and N1 contribute to NCoR binding by the RU486-ligated AR, consistent with a single NCoR molecule binding to the AR homodimer. However, interaction domain specificity is important as the interaction is not supported by N3, and may be dependent on N1.

To verify that the interactions between the RU486-ligated AR and the NCoR RIDs could occur directly, we employed a GST fusion protein containing N2 and N1, GST-NCoR (N2N1) and used it pull down a variety of AR moieties. To ensure proper folding of the AR, we transfected cells with the AR constructs used. As shown in Fig. 7C, GST-NCoR (N2N1) is able to recruit the DHT-ligated full-length AR without need of N3, which is consistent with the results shown in Fig. 7, A and B. Furthermore, the RU486-ligated AR was recruited more strongly, supporting the data derived from both one and two-hybrid assays. Significantly, the AR LBD alone was recruited by GST-NCoR (N2N1) and this recruitment was also enhanced by RU486. This result supports the conclusion that RU486 modifies the structure of the AR LBD to stimulate CoRNR box binding. However, this binding is apparently too weak to detect in reporter gene assays. Finally, we assessed the ability of the AR N-DBD to be recruited directly by GST-NCoR (N2N1). Indeed, a direct interaction was observed, suggesting that the surface of NCoR that recruits the AR N terminus lies in the N2N1 region (Fig. 7C).

AR N-terminal FXXLF Motif Is Not Required for NCoR Interaction with the RU486-ligated AR—The strong interaction between NCoR and the RU486-ligated full-length AR, but not the isolated RU486-ligated AR LBD in mammalian cells, indicates a critical role for the AR N terminus in NCoR binding. AR N-terminal binding to the agonist-ligated AR LBD is mediated primarily by an N-terminal LXXLL-like peptide, FQNL. Although the RU486-ligated AR LBD does not interact strongly with the AR N terminus in mammalian two-hybrid assays, this peptide may nonetheless contribute to stabilizing NCoR binding. Therefore, mutations in this peptide were examined for their effect on NCoR binding. The F23A mutation markedly impairs AR N-terminal interaction with the DHT-ligated LBD and substantially reduces DHT-stimulated transcriptional activity (Fig. 8, A versus B). However, this mutation...
did not impair VP16-NCoRc binding to the RU486-ligated AR. Moreover, it did not decrease the apparent high affinity for RU486 (with maximal interaction at between 1–10 nM) (Fig. 8B). The LF26,27AA double mutation, which also impairs N-C-terminal interaction and transactivation by the DHT-ligated AR, similarly failed to prevent NCoR binding by the RU486-ligated AR (Fig. 8C). Finally, deletion of all five residues (ARdel23–27) also failed to prevent AR interaction with VP16-NCoRc in the presence of RU486. Taken together, these results demonstrate that the AR N terminus contributes to NCoR binding via interactions that are distinct from those that mediate binding to the DHT-ligated AR LBD.

Previous studies have shown that the AR LBD can strongly repress a transactivation domain in the AR N terminus located between amino acids 360–528, termed transcription activation unit-5 (TAU-5), suggesting that this region may stabilize NCoR binding to the LBD (45, 46). Interestingly, an FXXLF-like motif (WHTLF) that can interact with the AR LBD is also located in this region of the N terminus (amino acids 433–437) (19). To determine whether NCoR binding was stabilized by this region
of the AR N terminus, we examined an AR(del1–366) mutant. Consistent with previous data, this mutant had substantially reduced transcriptional activity in response to DHT (Fig. 8E). However, neither the DHT nor the RU486-liganded AR(del1–366) were activated by VP16-NCoR. This result indicated that NCoR binding was not responsible for repression of AR(del1–366), and that site(s) N-terminal to amino acid 366 were required for NCoR binding.

**DISCUSSION**

The AR plays a critical role in prostate cancer development and progression, and hormonal therapies that ablate androgen action remain as critical mainstays of therapy. However, prostate cancers invariably become refractory to androgen ablation therapies, probably through diverse mechanisms that restore AR transcriptional activity despite the presence of castrate androgen levels or currently used AR antagonists (7, 8). We and others have previously demonstrated that the corepressors NCoR and SMRT can associate with the AR and inhibit AR transcriptional activity, but the roles these corepressors play in mediating AR responses to physiological agonists or to antagonists used for prostate cancer treatment have not been determined (24–27). This study confirmed the role of endogenous NCoR as a negative regulator of AR activity, and indicated that agonist-stimulated AR activity may be determined by the relative levels of NCoR versus coactivators. Significantly, while bicalutamide and other AR antagonists used for prostate cancer treatment could support AR recruitment of NCoR, RU486 was unique in its ability to markedly enhance the AR-NCoR interaction. This interaction was dependent on both the AR LBD and the N-terminal domain, the latter being independent of the FXXLF motif that mediates agonist-stimulated AR N-C-terminal interactions. Taken together, these results have identified a novel mechanism for AR recruitment of NCoR, which may be exploited for the development of potent AR antagonists with activity in early and potentially advanced prostate cancer.

The role of endogenous NCoR in AR action was shown by siRNA experiments, with NCoR down-regulation causing an increase in DHT-stimulated AR activity. Chromatin immunoprecipitation experiments in LNCaP prostate cancer cells further demonstrated DHT-dependent NCoR recruitment in vivo to both the PSA and p21 promoters. NCoR associates with HDAC3, and it seems likely that histone deacetlylase activity contributes to NCoR-mediated AR repression in vivo (47, 48). Nonetheless, we found previously that AR repression by transfected NCoR was dependent upon the C-terminal RIDs, and was not abrogated by deletion of the HDAC interacting N-terminal repressor domains. The data in this study show that an alternative mechanism of repression is through inhibition of coactivator binding to the AR N terminus. Significantly, a recent study also found DHT-dependent recruitment of TBL and TBLR1 to the PSA gene (49). These proteins form a complex with NCoR and HDACs, and transcriptional activation of other nuclear receptors is linked to proteasome-mediated degradation of this complex. Therefore, these data indicate that NCoR may function in recruitment of the TBL complex to the DHT-liganded AR, and that NCoR degradation may be required for full AR activation by DHT.

Previous studies using chromatin immunoprecipitation demonstrated that NCoR could be recruited to the PSA gene by bicalutamide (31, 32). This AR antagonist is widely used for prostate cancer treatment, but has very limited efficacy in the treatment of androgen independent disease. Significantly, we found that the AR-NCoR interaction was not enhanced by bicalutamide, or by a series of other antagonists. In contrast, the AR partial agonist RU486 markedly enhanced AR recruitment of NCoR in a series of mammalian one- and two-hybrid protein interaction experiments, using both an artificial multimerized ARE and endogenous AREs from the PSA gene. Moreover, NCoR siRNA enhanced the weak partial agonist activity of RU486, indicating that endogenous NCoR is recruited by the RU486-liganded AR and contributes to its antagonistic activity. Two recent studies have similarly found NCoR recruitment by the RU486-liganded AR, and previous studies have shown that RU486 can recruit NCoR to the PR and GR (13, 28, 41–43). The unique structural feature of RU486 is a bulky group in the 11β position, which appears to interfere with binding of coactivator LXXLL-motifs and allow for CoRNR box binding. Taken together, these studies demonstrate that the structure of the AR LBD can be altered by appropriate ligands to markedly enhance NCoR binding, and suggest that such ligands may function as more potent AR antagonists than bicalutamide or other AR antagonists currently in use for prostate cancer treatment.

Recruitment of NCoR to the DHT-liganded AR requires one or more of the three RIDs in the C terminus of NCoR, as mutations in all three RIDs abrogate NCoR repression of the DHT-liganded AR. The NCoR and SMRT RIDs share a common helical motif (LXXLH/LXXXL), but it is clear that differences among the domains allow for nuclear receptor specificity (34, 37, 50–53). The most N-terminal of the NCoR RIDs, N3, is required to recruit the TR. This RID is not present in SMRT and explains the preference of NCoR for the TR (34, 53). The middle NCoR RID, N2 prefers the TR but can also interact weakly with the RAR. The unique CoRNR box sequence present in the homologous domain in SMRT, S2 (ISEVI), allows SMRT to preferentially recruit the RAR (36, 37). The C-terminal domains in NCoR and SMRT, N1, and S1, share significant homology and have been shown to bind to RXR isoforms strongly and to be recruited to PPARα in the presence of an agonist (54). In this study we demonstrate that the recruitment of NCoR to the RU486-liganded AR requires the C-terminal N1 domain and is enhanced by N2, but is independent of N3. Furthermore, the interaction between N2 and N1 and the DHT- or RU486-liganded AR is mediated by a direct protein-protein interaction. These findings indicate that the AR homodimer may be similar to other nuclear receptor dimers, which recruit a single NCoR molecule via two RIDs. Preferential binding of N1 by the AR is supported by a recent study examining the recruitment of SMRT to the DHT-liganded AR, which found that the homologous S1 domain interacted most strongly in GST solution assays with the AR (26). Whether the additional preference for N2 instead of N3 is secondary to its closer proximity to N1 or to its sequence is not yet clear. Further mutational studies are underway to understand the structural mechanism by which separate NCoR RIDs are recruited to the AR, and these should aid in the rational design of new potent AR antagonists.

While the AR LBD is necessary for NCoR recruitment, our data show that the AR N terminus is also required for a strong AR-NCoR interaction. NCoR-mediated repression of the constitutive transcriptional activity of the isolated AR N-terminal domain supports a direct interaction between NCoR and the AR N terminus. This direct interaction is further supported by the ability of NCoR to directly bind to the AR N terminus in a protein-protein interaction assay. Moreover, NCoR strongly repressed AR coactivation by SRC-1 and -2, which interact primarily with the AR N-terminal domain. As an alternative to a direct interaction between NCoR and the AR N-terminal domain, NCoR binding to the AR LBD may instead be stabilized indirectly by an interaction between the AR N terminus and LBD. However, in contrast to the DHT-ligated AR LBD, the RU486-ligated AR LBD does not interact with the AR N terminus in mammalian
two-hybrid protein interaction assays. Furthermore, mutation or deletion of the FXXFL motif from the proximal AR N terminus does not impair NCoR binding to the RU486-ligated AR. Significantly, a recent study found that NCoR binding to the RU486-ligated GR was similarly dependent on the GR N terminus, and direct interactions between the AR N terminus and SMRT have been reported (25, 27, 55). Taken together, these studies indicate that NCoR is a physiological regulator of the agonist-ligated AR, and that the relative expression of NCoR versus AR coactivators may in part regulate distinct AR responses to androgens in different target tissues. This study further shows that the AR-NCoR interaction is not enhanced by AR antagonists used currently for the treatment of prostate cancer, but can be markedly enhanced by RU486. NCoR interaction with the RU486-ligated AR is mediated by the AR LBD and by a site in the AR N terminus. Importantly, a further consequence of the recruitment of NCoR and the AR N terminus by the RU486-ligated AR may be to stabilize RU486 binding, accounting for the activity of RU486 in the nanomolar range versus the micromolar range for bicalutamide. In any case, RU486 clearly represents a new class of AR antagonists that will likely have novel activities in vivo. Clinical trials of RU486 or related drugs are needed to determine whether these may be more efficacious than currently available AR antagonists in the treatment of prostate cancer, particularly advanced androgen independent prostate cancer.

Acknowledgments—We thank E. Wilson and Y. Shi for plasmids.

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