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Inhibition of the Dihydrotestosterone-Activated Androgen Receptor by Nuclear Receptor Corepressor

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Nuclear receptor corepressor (NCoR) mediates transcriptional repression by unliganded nuclear receptors and certain steroid hormone receptors (SHRs) bound to nonphysiological antagonists, but has not been found to regulate SHRs bound to their natural ligands. This report demonstrates that NCoR interacts directly with the androgen receptor (AR) and represses dihydrotestosterone-stimulated AR transcriptional activity. The NCoR C terminus, containing the receptor interacting domains, was necessary for repression, which was ablated by mutations in the corepressor nuclear receptor (CoRNR) boxes. In contrast, the NCoR N terminus, containing domains that can recruit histone deacetylases, was not necessary for repression. Binding studies *in vitro* with a series of glutathione-*S*-transferase-NCoR and -AR fusion proteins demonstrated a direct interaction that was similarly dependent upon the NCoR corepressor nuclear receptor boxes and AR ligand binding domain and was independent of ligand and helix 12 in the AR ligand binding domain. This NCoR-AR interaction was further demonstrated in mammalian two-hybrid assays and by coimmunoprecipitation of the endogenous proteins from a prostate cancer cell line. Finally, AR transcriptional activity could be enhanced *in vivo* by sequestration of endogenous NCoR with unliganded thyroid hormone receptor. These results demonstrate that AR, in contrast to other SHRs, is regulated by NCoR and suggest the possibility of developing selective AR modulators that enhance this interaction. (*Molecular Endocrinology* 16: 1492–1501, 2002)

THE ANDROGEN RECEPTOR (AR) is a steroid hormone receptor (SHR) member of the larger nuclear receptor (NR) superfamily of ligand-regulated and sequence-specific transcription factors and is critical for normal male development as well as prostate cancer development and progression (1–4). The AR and other unliganded SHRs associate with a heat shock protein 90 complex that functions as a chaperone to maintain the C-terminal ligand binding domain (LBD) in a conformation competent to bind ligand. Ligand binding causes a conformational change in the LBD with subsequent dissociation from heat shock protein 90, homodimerization, and DNA binding mediated by a central DNA binding domain (DBD). A major feature of this ligand-induced conformational change is movement of helix 12 in the LBD against helices 3 and 5, generating a small hydrophobic cleft that binds LXXLL motifs (where L is leucine and X is any amino acid) (5–11). These LXXLL motifs were identified initially in the p160 family of steroid receptor coactivator (SRC) proteins, which mediate the LBD transactivation function (AF-2) through intrinsic histone acetyltransferase activity and recruitment of CREB-binding protein/p300 (8, 10, 12–20). However, the LXXLL motif has been identified in multiple other transcriptional regulatory proteins that presumably compete for LBD binding.

Although the DBD and LBD of SHRs are highly conserved, there is much less homology among SHRs in the N-terminal domain. The AR has a particularly long N-terminal domain with a very strong autonomous transactivation function (AF-1) (21, 22). The AR and estrogen receptor α (ERα) N-terminal domains have been found to interact directly with their respective LBDs (23–28). In the case of the AR, this interaction is mediated by LXXLL-like sequences in the N terminus, which presumably compete with other LXXLL motif-containing proteins for binding to the LBD (28). The AR and ERα N-terminal domains can also bind directly to SRC-1 and SRC-2 (human transcriptional intermediary factor 2, murine glucocorticoid receptor interacting protein 1) through sites in the

Abbreviations: Ab, Antibody; AF-1, AF-2, transactivation functions 1 and 2; AR, Androgen receptor; ARE, androgen response element; CMV, cytomegalovirus; CoRNR, corepressor nuclear receptor; CS-FCS, charcoal dextran-stripped FCS; DBD, DNA-binding domain; DHT, dihydrotestosterone; E_2, estradiol; ERα, estrogen receptor-α; ERE, estrogen response element; FCS, fetal calf serum; GST, glutathione-*S*-transferase; HDAC, histone deacetylase; LBD, ligand-binding domain; NCoR, nuclear receptor corepressor; NR, nuclear receptor; 4OHT, 4-hydroxytamoxifen; RID, receptor interaction domain; SHR, steroid hormone receptor; SMRT, silencing mediator of retinoid and thyroid hormone receptor; SRC, steroid receptor coactivator; TR, thyroid hormone receptor; TSA, trichostatin A.

coactivators distinct from the LXXLL motifs (10, 27, 29–31). Indeed, SRC-1 binding to the AR appears to be primarily dependent upon the N terminus, as SRC-1 binding to the AR LBD is extremely weak or absent (10, 27, 31).

In contrast to SHRs, other NRs including thyroid hormone receptor (TR) isoforms, the retinoic acid receptor isoforms, and the vitamin D receptor, bind DNA and function as transcriptional repressors in the absence of ligand. This repression is mediated in part by two related corepressor proteins, the nuclear receptor corepressor (NCoR) (32) and the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) (33), which serve to repress transcription by recruiting histone deacetylases (HDACs) to target genes (34–37). Importantly, the corepressors interact with NRs through a binding site that overlaps the SRC site in the LBD. Binding is through conserved C-terminal receptor interaction domains (RIDs) in NCoR and SMRT. These RIDs have a hydrophobic core (I/LXXII, where I is isoleucine, L is leucine, and X is any amino acid), termed the corepressor nuclear receptor box (CoRNR box), that is related to the coactivator binding LXXLL motif (38–43). The ligand-induced movement of helix 12 in the LBD of NRs that generates the SRC binding site also occludes the NCoR and SMRT binding site and thereby acts as a corepressor-coactivator switch. Indeed, in some NRs such as retinoic X receptor, helix 12 functions as an inhibitor of corepressor binding even in the absence of ligand (44).

Certain synthetic partial agonists such as 4-hydroxytamoxifen (4OHT) for the ERα can induce an alternative positioning of helix 12 in the LBD that permits corepressor binding (6, 11, 45–47). Indeed, 4OHT functions as an ERα agonist in fibroblasts from NCoR knockout mice (48). NCoR binding to the 4OHT-liganded ERα may mediate the therapeutic effects of 4OHT in breast cancer, but the physiological significance of these corepressor binding interactions have been unclear as neither NCoR nor SMRT has been found to repress the activity of SHRs stimulated by their natural ligands. This report demonstrates that NCoR interacts with the AR and represses transcriptional activity stimulated by dihydrotestosterone (DHT), a natural ligand for the AR. These findings indicate that NCoR is a physiological regulator of AR transcriptional activity and support the development of novel AR antagonists that enhance the AR-NCoR interaction.

RESULTS

NCoR Represses AR Transcriptional Activity

The weak interaction between SRC-1 and the AR LBD, the lack of AR AF-2 activity in mammalian cells, and the reported AR AF-2 activity in yeast (49) suggested that the agonist-bound AR AF-2 might permit binding to a mammalian corepressor. This hypothesis was tested by cotransfection of AR and NCoR expression vectors, with an androgen response element (ARE_4)-luciferase reporter gene. NCoR, but not the parent PKCR2 vector, inhibited the DHT-induced transcriptional activation of the AR (Fig. 1A and data not shown). This inhibition did not reflect a nonspecific effect on transcription, as neither the basal activity of the ARE_4-luciferase reporter nor a control cytomegalovirus (CMV)-regulated Renilla reporter gene were decreased by NCoR (Fig. 1B). Furthermore, NCoR did not down-regulate AR protein levels in transfected cells (Fig. 1C). Finally, NCoR did not inhibit the transcriptional activity of the estradiol (E_2)-liganded ERα (Fig. 1D, *right panel*). Taken together, these results supported the hypothesis that NCoR interacted with and repressed the agonist-bound AR.

Repression Requires NCoR RIDs and Is Not HDAC Dependent

The N terminus of NCoR contains repressor domains (RD1–3) that function, at least in part, by recruiting HDACs (Fig. 2A). To determine whether NCoR repression of AR activity was HDAC dependent, transfections were carried out in the presence of trichostatin A (TSA), a specific HDAC inhibitor. TSA markedly enhanced overall transcriptional activity as well as DHT-stimulated AR transcriptional activity (Fig. 3A). However, this DHT-dependent AR activity in the presence of TSA was still strongly repressed by NCoR, suggesting that repression was not HDAC dependent.

To further address the HDAC requirement for NCoR repression of DHT-stimulated AR transcriptional activity, the function of an NCoR mutant with the HDAC binding N-terminal repressor domains deleted was assessed (NCoRI, see Fig. 2A) (43, 50). NCoRI similarly repressed AR transcriptional activity (Fig. 3B). An additional mutant ($NCoRI_d$), with a further deletion to remove a potential Sin3 binding site carboxy to the previously identified repressor domains, was also tested (Fig. 2A). Similarly to NCoRI, the $NCoRI_d$ mutant strongly repressed AR transcriptional activity (Fig. 3C). These findings indicated that repression was not dependent on HDAC or the N-terminal repression domains of NCoR.

It was next determined whether repression required the NCoR C-terminal RIDs previously shown to mediate binding to nonsteroid hormone nuclear receptors. The $NCoRI_m$ mutant contains the NCoR C terminus corresponding to NCoRI, but with point mutations in each of the NCoR CoRNR boxes (N1–3) that were shown previously to abrogate thyroid hormone receptor binding (43) (Fig. 2A). In contrast to the wild-type NCoRI, the $NCoRI_m$ mutant did not repress AR transcriptional activity (Fig. 3C). Importantly, immunoblotting showed that the lack of repression by $NCoRI_m$ was not due to lower protein expression as it was expressed at greater levels than NCoRI (Fig. 3D). Immunoblotting also demonstrated that transfection with NCoR mutants, similarly to intact NCoR, did not decrease AR protein levels (Fig. 3E). Taken together,

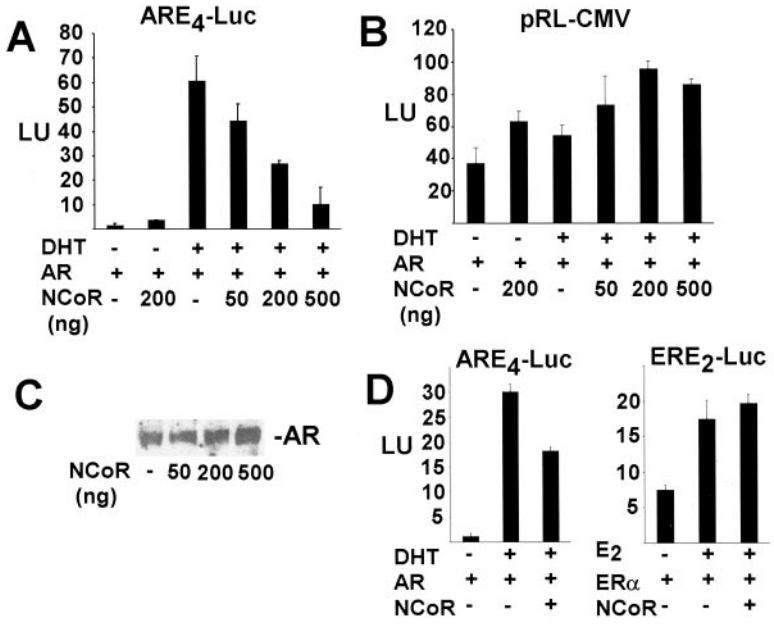

Fig. 1. NCoR Repression of AR Transcriptional Activity

A, CV-1 cells were cotransfected with 200 ng of ARE_4-luciferase reporter, 200 ng of AR expression vectors ($pSVAR_o$), 0.2 ng of pRL-CMV, and full-length NCoR expression vector (PKCR2-NCoR), as indicated. DHT (10 nM) was added as indicated 24 h after the transfection was initiated, and cells were harvested 24 h later. B, Renilla activity in the lysates from panel A. C, CV-1 cells were transfected as above in conjunction with the indicated amounts of NCoR expression vector, and 10 μl of the lysates (total 100 μl) were immunoblotted for AR expression. D, CV-1 cells were cotransfected with ARE_4- or ERE_2-luciferase reporters (200 ng), AR or ERα expression vectors (200 ng), pRL-CMV, and NCoR (200 ng) and treated with either 10 nM DHT or 10 nM E_2 as indicated.

these results demonstrated that the AR-NCoR interaction was mediated by the C-terminal RIDs. They further showed that the NCoR C-terminal RIDs could repress AR activity in the absence of N-terminal repressor domains, possibly through competition for coactivators or AR N- and C-terminal interactions.

AR-NCoR Binding *in Vitro* and *in Vivo*

Precipitation experiments using glutathione-*S*-transferase (GST)-NCoR fusion proteins corresponding to the above wild-type and RID mutants were next performed to further address whether there were direct binding interactions between the AR and NCoR (Fig. 4A). ^{35}S-Labeled AR generated by *in vitro* transcription/translation bound specifically to GST-NCoRI, containing the three C-terminal RIDs (Fig. 4B). Binding was not affected by DHT or by a pure AR antagonist, bicalutamide. However, GST-$NCoRI_m$, with mutations in the CoRNR boxes that abrogated repression of AR transcriptional activity, had diminished AR binding (~45% relative to GST-NCoRI).

The converse experiments were carried out using GST-AR fusion proteins (Fig. 2B). GST-AR(505–919), containing the AR DBD and LBD, bound specifically to ^{35}S-labeled NCoRI, with much weaker binding to the CoRNR box mutant $NCoRI_m$ (Fig. 4C). There was no binding to GST-AR(505–635), containing the AR DBD, whereas deletion of the AR C-terminal helices 9–12 in GST-AR(635–804) did not prevent binding (Fig. 4D). Taken together, these results supported a direct, helix 12-independent binding interaction between the AR LBD and the RIDs in NCoR.

Coimmunoprecipitation experiments were next carried out to determine whether there was an *in vivo* binding interaction between endogenous AR and NCoR. These experiments used the LNCaP cell line, the only generally available AR expressing human prostate cancer cell line. The AR expressed by LNCaP has a well characterized point mutation in the LBD (T877A), but it still responds to DHT (51). LNCaP cells were grown in medium with charcoal and dextran-stripped (steroid hormone-depleted) FCS (CS-FCS), plus or minus added DHT. Lysates were immunoprecipitated with anti-AR antibodies against the N or C terminus, and NCoR in the immunoprecipitates was detected with the affinity-purified polyclonal antibody (Ab) raised against a C-terminal NCoR peptide. Immunoblots of lysates from LNCaP cells demonstrated readily detectable levels of endogenous NCoR, which comigrated with transfected full-length NCoR (Fig. 5 and data not shown). NCoR was coimmunoprecipitated with the anti-AR N- and C-terminal antibodies, but not in the control immunoprecipitations (Fig. 5, A and B, respectively). There were no marked differences in the amount of NCoR coimmunoprecipitated with the AR from hormone-depleted or DHT-treated cells. These results demonstrated an interaction between NCoR and the AR *in vivo*, with comparable

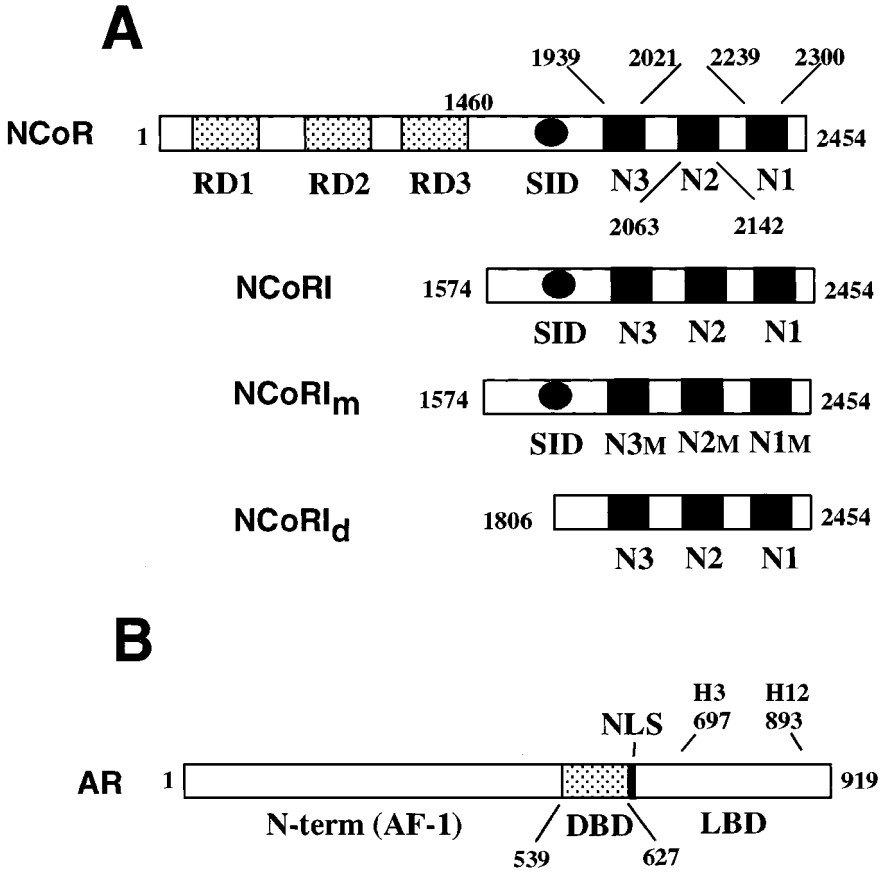

Fig. 2. Schematic Representation of Full-Length Human NCoR Deletion Mutants and AR

A, Full-length NCoR and mutants. RD1–3 are the N-terminal repressor domains, SID is a potential Sin3 interacting domain, and N1–3 are the C-terminal RIDs. NCoRI is a truncation to amino acid 1574, $NCoRI_d$ has a further deletion removing the SID, and $NCoRI_m$ has point mutations that substitute an alanine for the initial leucine or isoleucine in each of the three CoRNR boxes, as indicated. B, Outline of AR structure indicating the beginning of the DBD (residue 539), nuclear localization signal (NLS, residue 627), and helices 3 and 12 (residues 697 and 893, respectively).

amounts of AR-associated NCoR in the absence or presence of added DHT.

AR-NCoR Interaction in Mammalian Two-Hybrid Assays

Mammalian two-hybrid assays were carried out to further assess functional *in vivo* interactions between the AR LBD and the RIDs of NCoR. CV1 cells were cotransfected with the AR LBD (amino acids 660–919) fused to the Gal4 DBD [pBind-ARLBD(660–919)] and a fragment of NCoR corresponding to $NCoRI_d$ (amino acids 1806–2454), which was fused to the VP16 transactivation domain (AASVVP16-$NCoRI_d$). The Gal4-AR LBD(660–919) had no transcriptional activity, consistent with the weak or absent AF-2 of the AR LBD (Fig. 6A). However, transcriptional activity was strongly stimulated by cotransfection with the VP16-$NCoRI_d$ vector, in the presence or absence of DHT. In contrast, VP16-$NCoRI_d$ did not stimulate when coexpressed with the Gal4 DBD or Gal4-AR DBD vectors (Fig. 6B and data not shown). These results further supported a direct and ligand-independent interaction between NCoR and the AR LBD.

AR Transcriptional Repression by Endogenous NCoR

Experiments were next carried out to determine whether NCoR at physiological levels modulated ligand-dependent AR activity. Immunoblotting confirmed that CV1 cells expressed readily detectable levels of NCoR, which could be augmented by transfection (Fig. 7A). The unliganded TRβ, which binds to NCoR with high affinity (52), was used to sequester NCoR in cotransfection experiments with AR and an ARE_4-luciferase reporter gene. TRβ in the absence of ligand enhanced DHT-dependent AR activity approximately 2-fold (Fig. 7B). Addition of ligand for TRβ, T_3, abrogated this increase in AR transcriptional activity and resulted in repression below the level in the absence of TRβ. This result was consistent with a ligand-induced release of NCoR from the TRβ and subsequent competition for transcriptional coactivators.

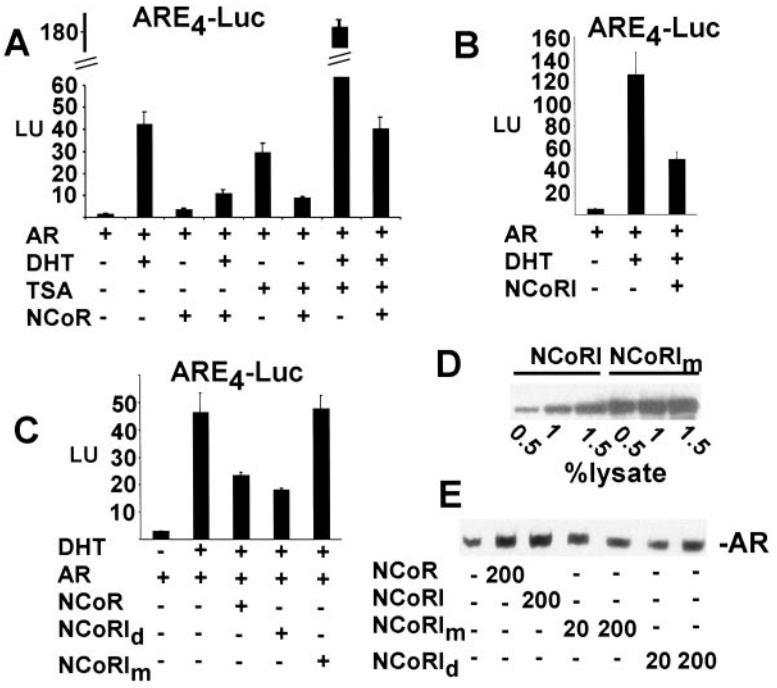

Fig. 3. NCoR Repression of AR Transcriptional Activity Mediated by the RIDs

A, CV-1 cells were cotransfected with pSVAR$_o$, ARE$_4$-luciferase, pRL-CMV, and full-length PKCR2-NCoR at 200 ng and were then treated with DHT (10 nM) or TSA (100 nM), as indicated. B, CV-1 cells were transfected with pSVAR$_o$, ARE$_4$-luciferase, pRL-CMV, and PKCR2-NCoRI (200 ng) and treated with DHT (10 nM) as indicated. C, CV-1 cells were transfected with pSVAR$_o$, ARE$_4$-luciferase, pRL-CMV, and PKCR2-NCoRI$_d$ or -NCoRI$_m$ expression vectors (200 ng) as indicated. D, Lysates from cells transfected as above with NCoRI or NCoRI$_m$ expression vectors were immunoblotted with an affinity-purified rabbit Ab generated against a C-terminal NCoR peptide. E, Lysates from cells transfected with the indicated amounts of NCoR expression vectors were immunoblotted for AR.

A further control was a TRβ hinge region mutant, TRβ(P214R), shown previously to be defective in NCoR binding (53). In contrast to the wild-type TRβ, the TRβ(P214R) mutant did not augment AR transcriptional activity. Taken together, these results indicated that endogenous NCoR functioned as a negative regulator of DHT-stimulated AR transcriptional activity.

DISCUSSION

Nonsteroidal NRs bind NCoR in the absence of ligand, but SHRs have been found previously to bind NCoR only in the presence of nonphysiological partial agonists such as 4OHT and raloxifene for the ERα (6, 11, 45–47). In contrast to these findings with other SHRs, this report demonstrates that NCoR binds to the AR and functions as a negative regulator of agonist-dependent AR transcriptional activity. Agonist ligand binding by most SHRs generates a high-affinity site for binding coactivator proteins through LXXLL motifs, accounting for the independent transcriptional activity of the LBD (AF-2). However, the AR LBD has minimal independent transcriptional activity, and this can be attributed to very weak SRC coactivator binding. The 4OHT- and raloxifene-liganded ERα assume conformations with helix 12 positioned away from helices 3 and 5, thereby failing to generate the high-affinity LXXLL coactivator binding site and revealing a cryptic corepressor binding site (6, 11). Taken together, these observations suggest the hypothesis that the DHT-liganded AR LBD may assume an alternative conformation with helix 12 positioned so that it does not ablate the NCoR binding site.

However, recent crystal structures of the agonist-liganded AR LBD do not demonstrate such alternative positioning of helix 12. Although the DHT-liganded AR LBD crystallized as a monomer, helix 12 was still positioned next to helices 3 and 5 as in the agonist-liganded ERα and PR (54). Helix 12 was split into two shorter helices when the AR was bound to the synthetic androgen R1881 (55), but these were again positioned similarly to helix 12 in agonist-bound ER and PR. Therefore, while it is possible that this agonist conformation of the AR can bind NCoR or that the AR in solution has a unique agonist conformation that can bind coactivators or corepressors, an alternative hypothesis consistent with the above structural data is that the agonist-bound AR can readily flip between coactivator and corepressor binding conformations. Each conformation would be stabilized *in vivo* by coactivator or corepressor binding, respectively, so the

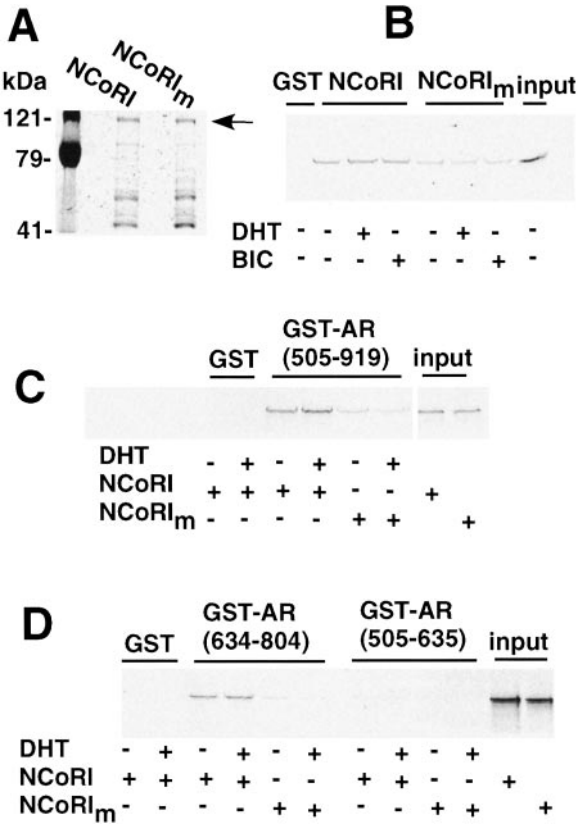

Fig. 4. NCoR Binding to the AR *in Vitro*

A, Coomassie blue-stained gel of GST-NCoRI and GST-$NCoRI_m$ fusion proteins, with *arrow* indicating the intact fusion proteins. B, GST, GST-NCoRI, and GST-$NCoRI_m$ fusion proteins (5 μg) on glutathione-agarose beads were incubated with 5 μl of ^{35}S-methionine-labeled AR, with DHT (10 nM) or bicalutamide (100 nM), as indicated, and bound protein was determined. Input represents 2 μl (40%) of the AR used for binding. C and D, The indicated GST or GST-AR fusion proteins (5 μg) bound to glutathione-agarose beads were incubated as above with 5 μl of *in vitro* transcription/translation generated ^{35}S-labeled NCoRI or $NCoRI_m$, with DHT (10 nM) as indicated. Input represents 2 μl (40%).

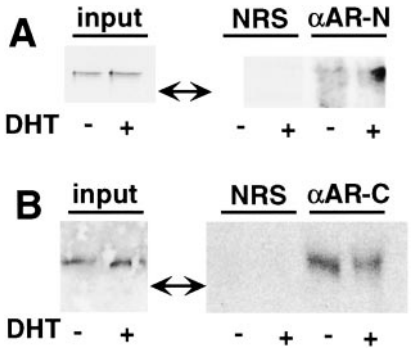

Fig. 5. Coimmunoprecipitation of Endogenous AR and NcoR

LNCaP cells grown to confluence on 10-cm plates in RPMI-164, 10% CS-FCS, and 10 nM DHT as indicated were lysed and immunoprecipitated with anti-AR N-terminal (A) or C-terminal (B) antibodies or normal rabbit serum (NRS) IgG, followed by anti-NCoR immunoblotting. *Double arrow* is position of 220-kDa marker. Input is 1% of the lysate.

relatively weak AR interaction with coactivators through LXXLL motifs could permit increased NCoR binding. Conversely, the lack of detectable NCoR interaction with other agonist-liganded SHRs presumably reflects stronger coactivator binding as well as enhanced agonist-induced stabilization of the LXXLL binding conformation. Importantly, the conformation of agonist-bound AR could be further regulated by posttranslational modifications, including phosphorylation and acetylation, which may modulate transcriptional activity (2, 56).

Whether the AR assumes a single agonist conformation that can bind NCoR or flips between discrete coactivator and corepressor binding conformations, NCoR binding would almost certainly prevent coactivator protein binding and would likely interfere with critical interactions between the N terminus and LBD mediated by N-terminal LXXLL-like motifs (27, 28). This interference may account for the transcriptional repression mediated by transfection of NCoR C-terminal RIDs lacking known repressor domains. Alternately, repression may be mediated by a recently identified corepressor that binds to the C terminus of SMRT through a sequence with homology to NCoR (57). In either case, the N-terminal repressor domains and their associated HDACs most likely contribute to repression of AR transcriptional activity on endogenous genes at physiological NCoR levels *in vivo*.

The physiological functions of most SHRs are regulated largely by the levels of their corresponding steroid hormones, although the responses to these hormones may certainly be modulated by other factors. In contrast, the biological activity of AR differs from other SHRs in that androgen levels do not fluctuate markedly in adult males. Therefore, the relative levels of NCoR and other corepressors (58) *vs.* coactivator activity may be more important regulators of AR function. NCoR may further provide a link between AR and other NRs, with increased NCoR activity and a subsequent decrease in AR function possibly contributing to the therapeutic effects in prostate cancer of retinoids, vitamin D, and troglitazone, the latter being a ligand for the peroxisome proliferator-activated receptor γ (59). Most significantly, the data presented here suggest the potential for development of selective AR modulators that would promote NCoR binding. Such drugs could be an alternative to androgen-ablative therapies in prostate cancer and could make important contributions to prostate cancer prevention and treatment.

MATERIALS AND METHODS

Plasmids and Reagents

Expression vectors for the AR ($pSVAR_o$) (60), ERα (pcDNA-ERα) (61), NCoR (PKCR2-NCoR) (32), TRβ (PKCR2-TRβ) (52),

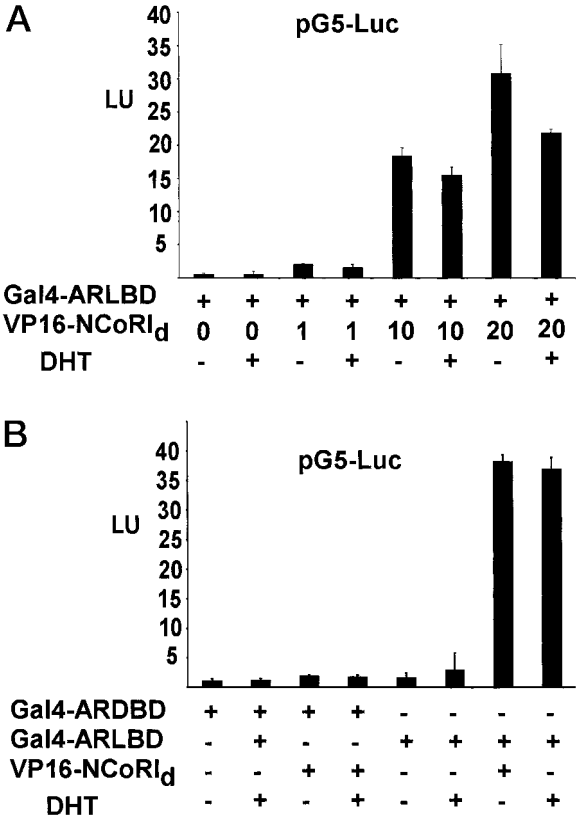

Fig. 6. NCoR-AR Interaction in Mammalian Two-Hybrid Assay

A, CV-1 cells were cotransfected with pG5-luciferase reporter (100 ng), pBind-ARLBD (100 ng), pRL-CMV, and AASVP16-NCoRI$_d$ (0–20 ng) and treated with DHT as indicated. B, CV-1 cells were cotransfected with pG5-luciferase reporter (100 ng), pBind-ARDBD or ARLBD (100 ng), pRL-CMV, and AASVP16-NCoRI$_d$ (0–20 ng) and treated as indicated.

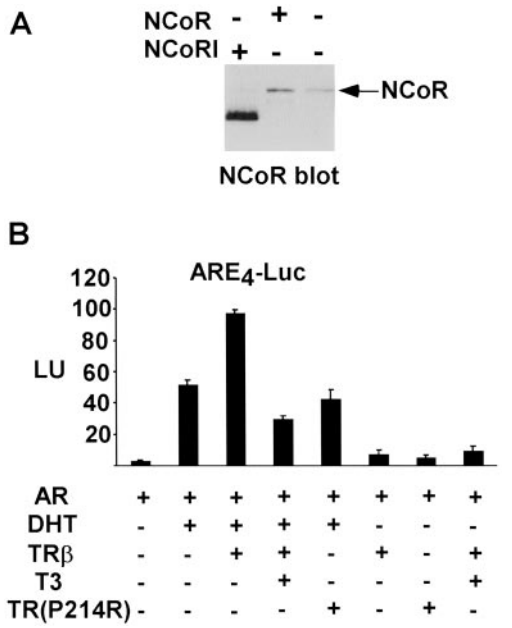

Fig. 7. AR Transcriptional Activity Enhanced by NCoR Sequestration

A, CV-1 cells were transfected as indicated with full-length (PKCR2-NCoR) or C-terminal (PKCR2-NCoRI) NCoR expression vectors, and lysates were immunoblotted with NCoR C-terminal antibody. *Arrow* indicates position of endogenous NCoR. B, CV-1 cells were transfected with pSVAR$_o$ (100 ng), ARE$_4$-luciferase (200 ng), pRL-CMV (0.2 ng), PKCR2-TRβ (200 ng), or PKCR2-TRβ(P214R) (200 ng) vectors, and cultured in DMEM and 10% CS-FCS with 10 nM DHT or 10 nM T_3, as indicated.

and TRβ(P214R) [PKCR2-TRβ(P214R)] (53) have been described. NCoRI is a truncation of human NCoR to amino acid 1574 that was initially cloned in a yeast two-hybrid screen (43, 50). NCoRI$_d$ has a further deletion to amino acid 1806 that removes a putative Sin3 interacting domain. NCoRI$_m$ was derived from NCoRI and has point mutations that substitute an alanine for the initial leucine or isoleucine in each of the three CoRNR boxes (43). The NCoR mutants were cloned into PKCR2 or AASVVP16 for mammalian expression and pGEX4T1 for GST fusion protein production (43). GST-AR fusion protein expression vectors in pGEX-2TK have been described previously (62). The AR LBD (amino acids 660–919) and DBD (501–660) were cloned into the mammalian Gal4 DBD fusion vector pBIND (Promega Corp., Madison, WI), to give pBIND-ARLBD(660–919) and pBIND-ARDBD(501–660), respectively. The reporter genes used were ARE$_4$-luciferase containing four tandem copies of a synthetic ARE (63), estrogen response element (ERE$_2$)-luciferase containing two EREs (61), a CMV-regulated Renilla control, pRL-CMV (Promega Corp.), and pG5-Luc (regulated by five tandem Gal4 binding sites) (Promega Corp.). TSA, DHT, E_2, and T_3 were from Sigma (St. Louis, MO).

Transfections

CV-1 cells in 24-well plates in DMEM with 10% CS-FCS (HyClone Laboratories, Inc., Logan, UT) were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and the indicated vectors, using 0.2 ng of pRL-CMV for normalization. Fresh medium was added after 24 h, and DHT (10 nM) or other hormones were then added for an additional 24 h. Firefly and Renilla luciferase activities were assayed with a Dual Luciferase Reporter Assay System Kit (Promega Corp.). Firefly luciferase activities in identically treated triplicate or quadruplicate samples were normalized for Renilla activities, and these values were used to determine standard deviations (SDS), with *error bars* indicating 1 SD. However, as some treatments had effects on the Renilla activities, the presented firefly luciferase results reflect the actual means of triplicate or quadruplicate samples that are not adjusted for differences in Renilla between experimental conditions. The data shown are representative of at least three independent experiments.

Immunoblotting

Cell lysates were immunoblotted for AR expression using pooled polyclonal rabbit Ab specific for the AR N- and C-termini (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Other lysates were immunoblotted with an affinity purified rabbit Ab generated against a C-terminal NCoR peptide (PHQQNRIWEREPAPLLSAQ) (Hollenberg, A., unpublished data).

Precipitations

GST, GST-NCoRI, and GST-NCoRI$_m$ fusion proteins (5 μg) bound to glutathione-agarose beads were generated as de-

scribed (43) and incubated with 5 μl of ^{35}S methionine-labeled AR generated by *in vitro* transcription/translation (TNT reticulocyte lysate, Promega Corp.), using a pcDNA3-AR template. Binding was for 2–4 h at 4 C in a total volume of 0.5 ml of binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.3% Nonidet P-40, and 20% glycerol), with DHT (10 nM) or bicalutamide (100 nM), as indicated. After four washes with binding buffer, the beads were boiled in 30 μl Laemmli reducing sample buffer and analyzed by SDS-PAGE followed by autoradiography. GST or GST-AR fusion proteins (5 μg) bound to glutathione-agarose beads were incubated as above with 5 μl of *in vitro* transcription/translation-generated ^{35}S-labeled NCoRI or $NCoRI_m$, with DHT (10 nM) as indicated.

For coimmunoprecipitations, LNCaP cells grown to confluence on 10-cm plates in RPMI-1640, 10% CS-FCS, with 10 nM DHT as indicated, were lysed in 1 ml cell lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, and 4 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride]. The lysates were split and immunoprecipitated with 2 μg of polyclonal rabbit anti-AR specific for the AR N- or C-termini (Santa Cruz Biotechnology, Inc.) or 2 μg of normal rabbit serum IgG bound to 10 μl of protein A-Sepharose beads for 2 h at 4 C. Proteins were run reduced on 6% SDS-PAGE and immunoblotted with the anti-NCoR peptide Ab.

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