The Partial Agonist Activity of Antagonist-Occupied Steroid Receptors Is Controlled by a Novel Hinge Domain-Binding Coactivator L7/SPA and the Corepressors N-CoR or SMRT

Twila A. Jackson, Jennifer K. Richer, David L. Bain, Glenn S. Takimoto, Lin Tung, and Kathryn B. Horwitz

Departments of Medicine and Pathology and the Molecular Biology Program
University of Colorado Health Sciences Center
Denver, Colorado 80262

Steroid receptor antagonists, such as the antiestrogen tamoxifen or the antiprogestin RU486, can have inappropriate agonist-like effects in tissues and tumors. To explain this paradox we postulated that coactivators are inadvertently brought to the promoters of DNA-bound, antagonist-occupied receptors. The human (h) progesterone receptor (PR) hinge-hormone binding domain (H-HBD) was used as bait in a two-hybrid screen of a HeLa cDNA library, in which the yeast cells were treated with RU486. We have isolated and characterized two interesting steroid receptor-interacting proteins that regulate transcription in opposite directions. The first is L7/SPA, a previously described 27-kDa protein containing a basic region leucine zipper domain, having no known nuclear function. When coexpressed with tamoxifen-occupied estrogen receptors (hER) or RU486-occupied hPR or glucocorticoid receptors (hGR), L7/SPA increases the partial agonist activity of the antagonists by 3- to 10-fold, but it has no effect on agonist-mediated transcription. The interaction of L7/SPA with hPR maps to the hinge region, and indeed, the hPR hinge region squelches L7/SPA-dependent induction of antagonist-mediated transcription. Interestingly, pure antagonists that lack partial agonist effects, such as the antiestrogen ICI164,384 or the antiprogestin ZK98299, cannot be up-regulated by L7/SPA. We also isolated, cloned, and sequenced the human homolog (hN-CoR) of the 270-kDa mouse (m) thyroid/retinoic acid receptor corepressor. Binding of hN-CoR maps to the hPR-HBD. mN-CoR, and a related human corepressor, SMRT, suppress RU486 or tamoxifen-mediated partial agonist activity by more than 90%. This suppression is completely squelched by overexpression of the hPR H-HBD. Additionally, both corepressors reverse the antagonist-dependent transcriptional up-regulation produced by L7/SPA. Our data suggest that the direction of transcription by antagonist-occupied steroid receptors can be controlled by the ratio of coactivators to corepressors recruited to the transcription complex by promoter-bound receptors. In normal tissues and in hormone-resistant breast cancers in which the agonist activity of mixed antagonists predominates, steroid receptors may be preferentially bound by coactivators. This suggests a strategy by which such partial agonist activity can be eliminated and by which candidate receptor ligands can be screened for this activity. (Molecular Endocrinology 11: 693–705, 1997)

INTRODUCTION

Steroid hormone antagonists, such as the antiestrogen tamoxifen or the antiprogestin RU486, are synthetic pharmaceutical agents that have been found empirically to suppress the activity of natural steroidal agonists such as estradiol, progesterone, or glucocorticoids (1–3). The ability of antagonists to suppress the transcriptional effects of agonists has important clinical value (2, 4). However, in some tissues and tumors, instead of being inhibitory, steroid antagonists can have inappropriate, agonist-like effects (4–8). The precise mechanisms by which antagonists inhibit tran-

0888-8809/97/$3.00/0
Molecular Endocrinology
Copyright © 1997 by The Endocrine Society

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

scription under some conditions, but stimulate it in others, are unknown (7).

Steroid hormones and their synthetic analogs bind to steroid receptors, which are members of a ligand-regulated family of nuclear transcription factors that includes, in addition to estrogen (ER) and progesterone (PR) receptors, the receptors for androgens, glucocorticoids (GR), and mineralocorticoids (9–11). These receptors belong to a distinct subgroup of the nuclear receptor superfamily, another subgroup of which includes the receptors for retinoic acids, vitamin D, and thyroid hormone (10). A key functional difference between steroid receptors and retinoic acid/thyroid hormone receptors is that the latter are constitutive transcriptional repressors, which bind to their cognate DNA-binding sites in the absence of ligand (12–14). In contrast, unliganded steroid receptors have little or no intrinsic DNA-binding ability or biological activity (12, 15). Instead, they require a ligand — either agonist or antagonist — to facilitate receptor-DNA interactions. The mechanisms by which unliganded retinoic acid/thyroid hormone receptors repress transcription were unknown until several recent studies described a new category of modulatory nuclear proteins having corepressor activity, which interact with the DNA-bound receptors and actively silence transcription (14, 16–22). Addition of ligand destabilizes corepressor binding to these receptors and activates transcription. These corepressors have been found to interact specifically only with unliganded members of the retinoic acid/thyroid hormone receptor subfamily, and they reportedly fail to interact with either unliganded or agonist-liganded members of the steroid receptor family (17, 19). No relationship has been known to exist between the mechanisms by which unliganded retinoic acid/thyroid hormone receptors repress transcription and the mechanisms by which antagonist-occupied steroid receptors inhibit the actions of agonists.

Little is known about the mechanisms by which steroid antagonists inappropriately activate transcription, although several models have been proposed. Partial agonist activity is often promoter- and cell type-specific (23, 24), and recently, a number of studies have shown that cross-talk between antagonist-occupied steroid receptors and cell surface-signaling pathways, such as activation by cAMP (4, 6, 25, 26), enhances these partial agonist effects, suggesting that unique receptor phosphorylation states mediate this activity.

We speculated that an alternative mechanism operates, namely that unique coactivator proteins are brought to the transcription complex by antagonist-occupied steroid receptors. To address this possibility, we used a LexA-human (h) PR hinge (H)-hormone binding domain (HBD) fusion protein as bait in a yeast two-hybrid screen of a HeLa cell cDNA library (27, 28). The assay incorporated a novel strategy in which the yeast cells were treated with the antiprogestin RU486. Using this screen, we have isolated two interesting proteins that regulate antagonist-occupied steroid receptors in opposite directions.

The first of these, L7 (29–36), is a 27-kDa cytoplasmic and nuclear protein believed to function in translational regulation (33) but of unknown nuclear function, which contains a canonical RNA- and DNA-binding leucine zipper bZIP dimerization domain (31). We find that human L7 or SPA (switch protein for antagonists) is a coactivator that strongly enhances transcription of RU486-occupied hPR or hGR, and tamoxifen-occupied hER, but has no effect on agonist-dependent transcription by these receptors. The second isolate is the human homolog (hN-CoR) of the mouse (m) retinoic acid/thyroid hormone receptor corepressor, N-CoR (17). Both mN-CoR and the related human corepressor SMRT (silencing mediator for retinoid and thyroid hormone receptors) (19), suppress the agonist-like transcriptional activity of RU486-occupied hPR and tamoxifen-occupied hER. Transcriptional repression mediated by N-CoR is reversed by L7/SPA.

We propose that the inhibitory pharmacological effects of antagonist-occupied steroid receptors involves the adventitious recruitment of a transcriptional corepressor that has no normal physiological function in steroid hormone action, whereas the partial agonist-stimulatory effects of antagonists involves recruitment of novel coactivators. Thus, the ratio of corepressors to coactivators that are bound to the transcription complex through the antagonist-occupied receptors determines whether the outcome is inhibitory or stimulatory. This property of antagonist-occupied receptors has therapeutic implications and suggests methods by which candidate receptor ligands can be screened for their partial *vs.* pure antagonist pharmacology.

RESULTS

Yeast Two-Hybrid Screening Strategy

To isolate proteins that interact with antagonist-occupied steroid receptors, we first asked whether agonists and antagonists have the appropriate transcriptional responses in yeast cells (Fig. 1A). L40 cells were transformed with a fragment of hPR (37, 38) consisting of the hinge region (H) and hormone binding domain (HBD) (amino acids 638–933) fused to LexA (pLexA: H-HBD). The cells were then treated with 1 μM progestin R5020, or 10 μM type II antiprogestins RU486 or ZK112993, or the type I antiprogestin ZK98299 as shown. R5020 strongly activates β-galactosidase transcription from the LexA operator, whereas the antagonists alone have no effect. However, RU486 and ZK112993 completely abolish R5020-dependent transcription, while ZK98299, which has a lower binding affinity for hPR, was 85% inhibitory. We conclude that appropriate antiprogestin-regulated transcriptional inhibition can be elicited in yeast cells.

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

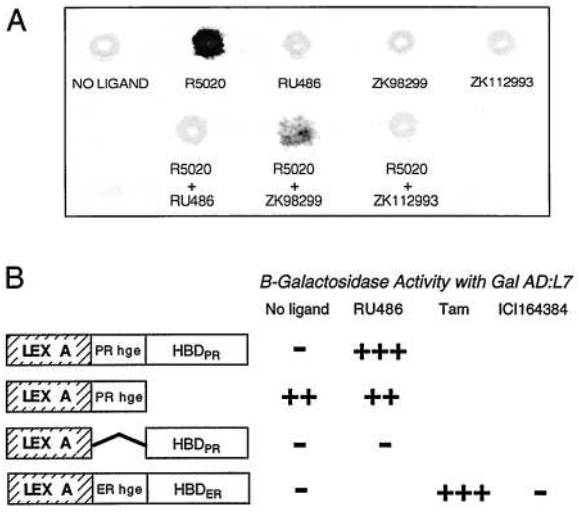

Fig. 1. L7/SPA, a Novel Protein That Interacts with Antagonist-Occupied Steroid Receptors

A, Antiprogestins can inhibit transcription by R5020 in yeast. The yeast two-hybrid strain L40 carrying a LexA promoter-LacZ reporter was cotransformed with an expression vector encoding the hPR hinge and hormone binding domain (hge, HBD) fused to LexA. Yeast cells were treated 48 h at 30 C with 1 μM of the agonist R5020, 10 μM of the three antiprogestins shown, either alone or in combination. Yeast colonies were lifted on a nitrocellulose filter and lysed, and a β-galactosidase assay was performed. B, The L7/SPA interaction maps to the hPR hinge region. The yeast two-hybrid strain L40 was cotransformed with the hPR or hER expression vectors encoding the fusion proteins shown in the figure and the vector encoding the Gal4 activation domain:L7/SPA fusion protein recovered from the library. Transformed yeast cells were grown for 2 days at 30 C on media containing 10 μM of the indicated antagonists. Colonies were lifted on a nitrocellulose filter and lysed, and a β-galactosidase assay was performed.

To identify proteins that mediate the agonist and/or antagonist activity of antiprogestins, pLexA:H-HBD was then used as bait to screen a HeLa cell cDNA library in yeast cells exposed to 10 μM RU486. Approximately 10 million recombinants were screened, of which 28 clones, identified as unique by restriction mapping, were classified as positive in preliminary assays.

The Coactivator, L7/SPA

One clone, TJ48, interacted with the hPR H-HBD but not with a lamin bait, and had no intrinsic transcriptional activity in the GAL4 AD library vector (data not shown). TJ48 was sequenced and found to be identical to nucleotide (nt) 54 to 744 of the L7/SPA (35) cDNA, which encodes a 27-kDa protein originally defined as a potent autoantigen associated with the large ribosomal subunit (32, 34).The N terminus of L7/SPA contains a basic region leucine zipper (bZIP) domain (39, 40), through which it forms stable homodimers that bind to RNA and double-stranded DNA (31, 35). The protein is detectable in the cytoplasm and nuclei but not nucleoli of human cell lines (34), and the transcript is expressed in a variety of adult mouse tissues and in human T47D and HeLa cell lines (data not shown). It has no known nuclear function.

Full-length L7/SPA cDNA was isolated by RT-PCR from HeLa cell RNA and cloned into the pGEX 4T1 glutathione-S-transferase (GST) plasmid and into the pSG5 mammalian expression vector. The interaction between L7/SPA and the hPR H-HBD was confirmed by GST pull-down (data not shown) of *in vitro* translated L7/SPA. To further map the L7/SPA-hPR interaction, hPR H-HBD, H, or HBD/LexA bait fusion proteins were expressed in yeast cells together with the original GAL4 AD-L7/SPA library fusion protein, which lacks 18 N-terminal amino acids. The cells were treated or not with RU486, and β-galactosidase activity driven by the LexA promoter was measured (Fig. 1B). As shown, transcriptional activity in the presence of H-HBD is dependent on treatment of the cells with RU486. β-Galactosidase activity is entirely absent, however, in the presence of HBD alone, regardless of hormone treatment, and is constitutively active in the presence of the hinge domain. This suggests that L7/SPA binds to H, but that it is ordinarily blocked by the HBD, and that this inhibition can be relieved by RU486 occupancy of the HBD. Similarly, L7/SPA binding to the H-HBD of hER is dependent on occupancy by the antiestrogen tamoxifen, whereas the pure antiestrogen, ICI I64,384 (3), does not promote interactions between L7/SPA and hER.

To test the effect, if any, of L7/SPA on steroid receptor-mediated transcription in mammalian cells, a PRE_2-$TATA_{tk}$-CAT reporter was transfected into HeLa cells, and dexamethasone (Dex) or RU486-regulated transcription from the endogenous GR was measured (Fig. 2) in the absence or presence of exogenous full-length L7/SPA. Dexamethasone strongly induces transcription (lane 2) which is unaltered by overexpression of L7/SPA (lane 3). RU486 behaves as a partial agonist under these conditions (compare lanes 1 and 4). Surprisingly, the agonist activity of RU486 is enhanced 10-fold by overexpression of L7/SPA (lane 5), and this extensive up-regulation can be completely squelched by the hPR hinge domain (lane 6). Similar results are observed in HeLa cells transiently overexpressing recombinant hGR (lanes 7–12). Thus, L7/SPA appears to have the astonishing ability to strongly enhance the partial agonist activity of a steroid antagonist, without altering agonist-dependent transcription.

RU486 is a type II antiglucocorticoid/antiprogestin that promotes receptor-DNA interactions (41, 42) and has partial agonist activity. Type I antagonists, such as the antiprogestin ZK98299, lack this activity (42, 43). The effect of L7/SPA on ZK98299-regulated transcription was tested using a construct consisting of a truncated hPR (DBD-H-HBD), lacking the N terminus. As shown in Fig. 3A, the hPR C terminus (or DBD-H-HBD) is strongly activated by R5020 (lanes 3 and 4) which is not modified by L7/SPA (lane 4). On the other hand,

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

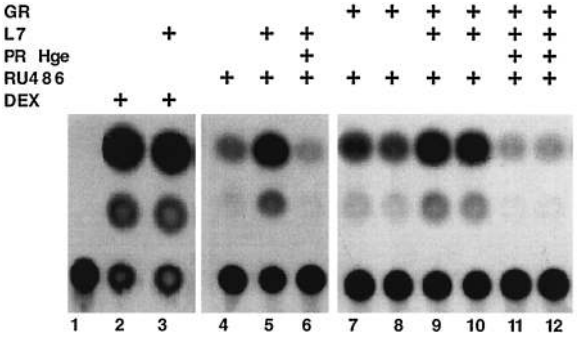

Fig. 2. L7/SPA Enhances the Partial Agonist Activity of RU486- but Not Dexamethasone-Mediated hGR Transcriptional Activity and This Activity Is Squelched by the hPR Hinge Region

HeLa cells were cotransfected with 2 μg PRE_2-$TATA_{tk}$-CAT reporter, with (lanes 7–12) or without (lanes 1–6) 10 ng hGR expression vector, and 5 μg full-length L7/SPA, plus or minus 5 μg hPR hinge expression vector as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were either untreated or treated with 100 nM RU486 or 10 nM dexamethasone for another 24 h. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC.

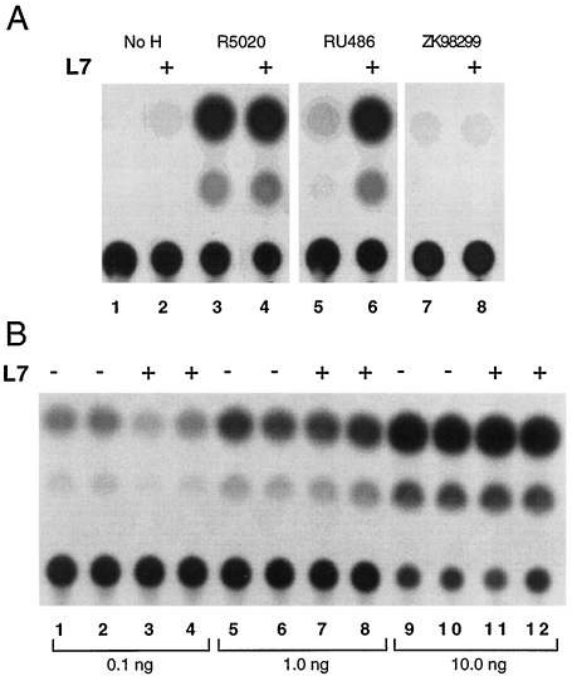

Fig. 3. L7/SPA Stimulates the Transcriptional Activity of the RU486-Occupied but Not the ZK98299- or R5020-Occupied hPR

A, HeLa cells were cotransfected with 2 μg PRE_2-$TATA_{tk}$-CAT reporter, 10 ng hPR C-terminus consisting of the DNA binding domain linked to the hinge region and HBD, and 5 μg full-length L7/SPA expression vectors as indicated in the figure (+). Twenty-four hours after transfection, the medium was changed, and the cells were either untreated or treated with 100 nM RU486 or ZK98299 or 10 nM R5020. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC. B, HeLa cells were transfected as above with 0.1, 1.0, and 10 ng full-length hPR B-receptors and treated with 10 nM R5020.

the partial agonist activity of RU486 (lane 5) on the PR C terminus is strongly enhanced by L7/SPA (compare lanes 5 and 6). In contrast, ZK98299 lacks partial agonist activity (lane 7) and is unaffected by L7/SPA (lane 8). Full-length hPR B-receptors have the same properties (not shown) as do hPR specificity mutants (43) in which the PRE-binding specificity of the PR DBD has been switched to an estrogen response element by mutation of three key amino acids (44, 45) in the first zinc finger (data not shown). This was done to rule out any effects of endogenous GR, on recombinant hPR-regulated transcription.

The data in Fig. 3A show that L7/SPA influences antagonist- but not agonist-mediated transcription. To analyze its effects on the agonist in more detail, submaximal concentrations of hPR B-receptors were tested (Fig. 3B). HeLa cells were transfected with the reporter, with 0.1, 1.0, and 10.0 ng of the B-receptor expression vector in the presence or absence of the L7/SPA expression vector, and the cells were treated with saturating concentrations of R5020. At 0.1 and 1.0 ng DNA, B receptor expression and its ability to transactivate PRE_2-$TATA_{tk}$-CAT are submaximal, yet L7/SPA is unable to enhance transcription. These data suggest that the effects of L7/SPA are indeed antagonist-specific.

Depending on their structure, some antiestrogens (tamoxifen, for example) possess partial agonist activity, whereas other antiestrogens (such as ICI 164,384) do not (3). To determine whether L7/SPA modifies the effects of antiestrogens, HeLa cells were transfected with wild type hER and the ERE_2-$TATA_{tk}$-CAT reporter in the presence or absence of the L7/SPA expression vector and were either left untreated or treated with 17β-estradiol (Fig. 4A) or the antiestrogens shown (Fig. 4B). Estradiol-dependent hER-mediated transcription is not influenced by L7/SPA even under submaximal hER expression levels (Fig. 4A). In contrast, the partial agonist activity of tamoxifen is further enhanced by L7/SPA overexpression (Fig. 4B). This increase can be squelched (46) by expression of the hPR hinge region. The extent of L7/SPA squelching by the hPR hinge can not be gauged without extensive titration studies, but we find that hPR hinge overexpression can reduce the partial agonist effect of tamoxifen even in the absence of L7/SPA (Fig. 4B), suggesting that endogenous cellular coactivators can also bind the hinge domain. On the other hand, like ZK98299, the antiestrogen ICI 164,384, which lacks partial agonist activity, is unaffected by L7/SPA. Thus, the activity of steroid antagonists that have partial agonist activity can be further enhanced by expression of L7/SPA, while pure antagonists and agonists are unaffected by this unusual coactivator. This explains the failure of ICI 164,384 to promote hER interaction with L7/SPA in the yeast two-hybrid screen (Fig. 1B).

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

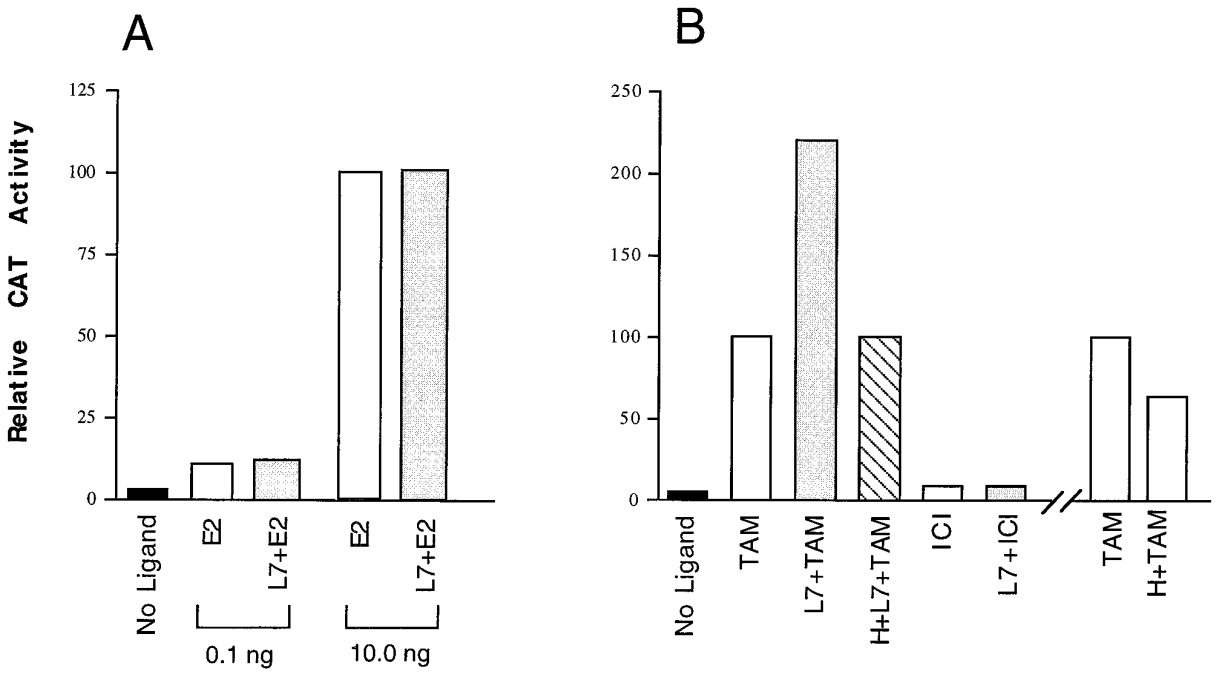

Fig. 4. L7/SPA Enhances the Partial Agonist Effects of Tamoxifen but Has No Effect on Estradiol or the Pure Antagonist ICI 164,384

A, HeLa cells were cotransfected with 2 μg ERE_2-$TATA_{tk}$-CAT reporter and 0.1 or 10 ng hER with or without 5 μg L7/SPA as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were either untreated or treated with 10 nM 17β-estradiol for another 24 h. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). B, HeLa cells were transfected as above, in the presence or absence of 5 μg hPR hinge (H) expression vector as indicated, and the cells were either untreated or treated with 100 nM tamoxifen or ICI 164,384. In panel A, estradiol-mediated CAT activity was set at 100% and in panel B, tamoxifen-mediated CAT activity was set at 100%. The agonist activity of tamoxifen is 50% that of estradiol.

The Corepressors N-CoR and SMRT

A second cDNA clone, TJ53, was isolated by yeast two-hybrid screening using RU486-occupied hPR H-HBD as bait (Fig. 5). Sequence analysis showed that this 3300-nt human cDNA (hN-CoR ID) was homologous to RIP13 (21), which is the interaction domain (ID) and surrounding C-terminal sequences of the 270-kDa mouse nuclear receptor corepressor (mN-CoR). Mouse N-CoR mediates ligand-independent repression by the thyroid hormone/retinoic acid receptor subfamily (17). Treatment with thyroid hormone or retinoic acid dissociates mN-CoR from the cognate receptors. It is noteworthy that the evidence presented in these studies indicates that mN-CoR does not interact with steroid receptors.

Using the hN-CoR ID cDNA recovered from the HeLa cell library cloned into the yeast vector, a HeLa cell cDNA cell library cloned into a bacterial vector was screened and, together with RT-PCR of HeLa and T47D cell RNA, the entire human coding sequence was obtained, sequenced, and compared with the murine protein-coding sequence (Fig. 5). In addition to a 7359-nt open reading frame that predicts a 2453-amino acid protein (hN-CoR1), two apparent N-terminal splice variants that result in loss of amino acids 83–206 (hN-CoR2) and amino acids 83–147 (hN-CoR3) in the N-terminal repressor domain (17) have been detected for the human corepressor. The amino acid identity between mN-CoR and hN-CoR1 is high (98.91%), with the greatest divergence observed in the second repressor domain (RD2) in which the identity falls to 80.4%. Because of this identity, we have used mN-CoR in most studies described below.

We find that the hN-CoR ID interacts with and modulates the activity of RU486-occupied hPR H-HBD and tamoxifen-occupied hER H-HBD. As shown in Fig. 6, the LexA/hPR fusion bait proteins were coexpressed in yeast cells together with the GAL4-AD/hN-CoR ID in the absence or presence of three antiprogestins. β-Galactosidase activity was dependent on the presence of RU486 when either the H-HBD or the HBD constructs were present in the cells, but it was uninducible with the H construct, suggesting that the hN-CoR ID interacts with the hPR HBD but not the hinge domain. This interaction is promoted by RU486 and by another type II antiprogestin, ZK112993, but not by the type I antiprogestin ZK98299. The latter is a pure antagonist that appears to inhibit hPR interactions with DNA (42, 47). Similarly, the interaction between the hER H-HBD and hN-CoR ID is very strong with tamoxifen occupancy, but minimal with the pure antiestrogen, ICI 164,384.

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

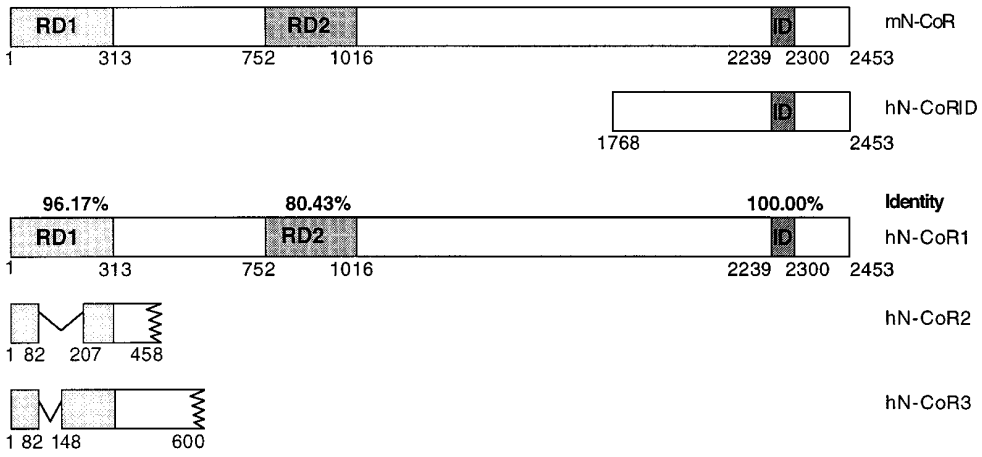

Fig. 5. A Comparison of the Mouse and Human N-CoR

The mN-CoR, as reported by Hörlein *et al.* (17) is shown at the top including two repressor domains (RD) at the N terminus and an interaction domain (ID) at the C terminus. The HeLa cell library clone isolated by yeast two-hybrid screen, TJ53, is referred to herein as hN-CoR ID. Also shown is the full-length hN-CoR1, together with its percent amino acid identity to mN-CoR. Two other clones contain deletions in RD1: hN-CoR2, an independent isolate from the HeLa cDNA bacterial library, lacks amino acids 83–206; hN-CoR3, an expressed tag sequence (Genbank accession number N33258), lacks amino acids 83–147. The amino acid sequence and protein structure downstream of amino acid 458 for hN-CoR2 and amino acid 600 for hN-CoR3 are unknown. Numbers refer to amino acids.

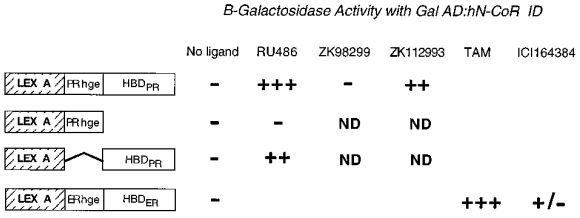

Fig. 6. The hN-CoR ID Interacts with the HBD of Steroid Receptors Occupied by Antagonists That Have Partial Agonist Activity, but Not to Pure Antagonists

The yeast two-hybrid strain L40 was cotransformed with the hPR or hER expression vectors encoding the fusion proteins shown in the figure and the vector encoding the Gal4 activation domain:hN-CoR ID fusion protein recovered from the library. Transformed yeast cells were grown for 2 days at 30 C on media containing no hormone addition or 100 μM of the indicated antagonists. Colonies were lifted on a nitrocellulose filter and lysed and a β-galactosidase assay was performed.

To characterize the function of the full-length corepressor on steroid receptors, we used mN-CoR (17) and the related corepressor SMRT, a 168-kDa protein described by Chen and Evans (19), having 48% identity to RIP13 (21), the C terminus of N-CoR. Similar to mN-CoR, the inhibitory properties of SMRT are restricted to the unliganded thyroid hormone/retinoic acid receptors. Figure 7 shows that either mN-CoR or SMRT suppresses the partial agonist activity of steroid antagonists. COS cells were transfected with the full-length hPR or hER expression vectors, expression vectors for mN-CoR or SMRT, the appropriate chloramphenicol acetyl transferase (CAT) reporter, and treated with RU486 or tamoxifen. As shown, the partial agonist activity of both steroid antagonists was more than 90% suppressed by either corepressor. This repression by mN-CoR or SMRT of the partial agonist effect of tamoxifen can be entirely squelched by coexpression of the hPR H-HBD (Fig. 8). Figure 8 also shows that the hPR H-HBD alone partially suppresses the agonist activity of tamoxifen, indicating that, in the absence of overexpressed N-CoR, other factors, possibly coactivators, may bind to the H-HBD.

Both N-CoR and SMRT are large proteins whose functional domains have not yet been well characterized. We find, for example, that the hN-CoR ID contains a transcriptional activation function that localizes to the C-terminal 240 amino acids (data not shown). Thus, these proteins may have functions other than the repressor ones described previously (17, 19). We also find (data not shown) that mN-CoR subtly suppresses, while SMRT subtly increases, the level of basal transcription from the promoters used in the present studies. This difference may be reflected in the data shown in Fig. 9, which show subtle effects of the corepressors on steroid receptor agonist-dependent transcription. In this study COS cells were transfected with expression vectors for hER, hPR A-receptors, and hPR B-receptors lacking 42 C-terminal amino acids (BΔ42). This truncation converts RU486 into an agonist (48). Cells were treated with the appropriate agonists (or RU486 in the case of BΔ42) in the presence or absence of SMRT or mN-CoR. We find that under these conditions of agonist-dependent transcription, SMRT consistently slightly up-regulates transcription (which rises to 3-fold in the case of BΔ42 and RU486), whereas mN-CoR consistently slightly decreases transcrip-

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

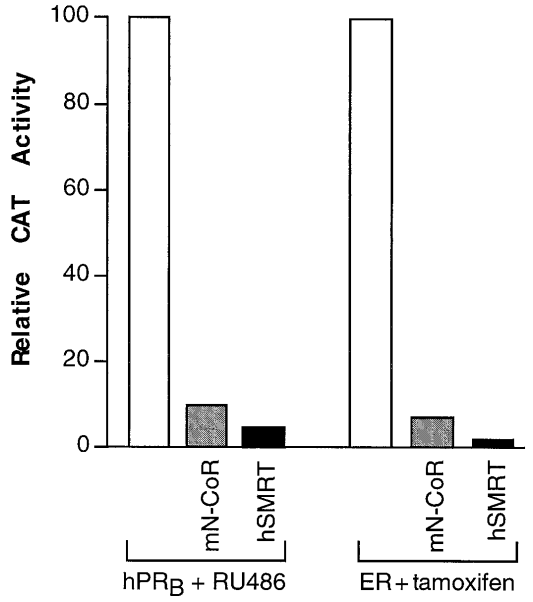

Fig. 7. Mouse N-CoR and Human SMRT Repress the Partial Agonist Activity of RU486-Occupied hPRB and Tamoxifen-Occupied hER

COS cells were cotransfected with 2 μg PRE$_2$-TATA$_{tk}$-CAT or ERE$_2$-TATA$_{tk}$-CAT reporters, 10 ng hPRB or hER, and 5 μg mN-CoR or hSMRT expression vectors as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and cells were either untreated or treated with 100 nM RU486 or tamoxifen. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantitated by phosphorimaging setting the partial agonist activity of RU486 or tamoxifen (*open bars*) at 100%.

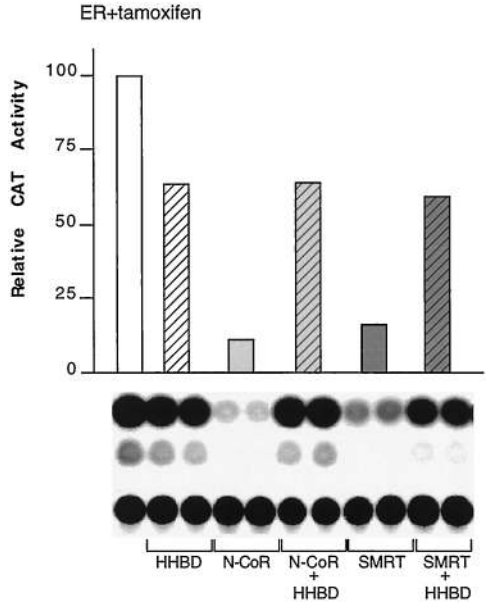

Fig. 8. Mouse N-CoR and hSMRT Suppress the Partial Agonist Activity of Tamoxifen-Occupied hER and the Effect of These Corepressors Is Squelched by the hPR H-HBD

COS cells were cotransfected with 2 μg ERE$_2$-TATA$_{tk}$-CAT reporter, 10 ng HEGO with or without 5 μg mN-CoR or hSMRT, and 5 μg hPR H-HBD expression vectors as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were treated with 100 nM tamoxifen for 24 h. Cell lysates were normalized to β-galactosidase activity, CAT assays were performed by TLC, and duplicate experimental points were quantitated by phosphorimaging and averaged. The partial agonist activity of tamoxifen-occupied hER was set at 100% (*open bar*). In this study, tamoxifen had 47% the agonist activity of estradiol (not shown).

tion. These subtle effects of the corepressors with agonists are in sharp contrast to the major inhibitory effects of both corepressors with antagonists (see Figs. 7 and 8). We ascribe these agonist-dependent effects of the corepressors to the influence that they appear to have on basal transcription levels of the promoters used in the present studies, but other explanations are possible. In contrast, L7/SPA has no effect on basal transcription levels and modulates only antagonist-dependent transcription.

The L7/SPA Coactivator Plus the Corepressors?

Because coactivators and corepressors appear to "coexist" in cells, it seems reasonable to propose that the ultimate direction of transcription under control of antagonist-occupied steroid receptors depends on the relative cellular levels of these two, offsetting classes of coregulatory proteins. The preliminary study in Fig. 10 suggests that this is indeed the case. Here, HeLa cells were transfected with an hGR expression vector alone, or together with L7/SPA or SMRT (either alone or in combination), and the cells were treated with RU486. As shown, L7/SPA alone enhances RU486-dependent transcription from the PRE$_2$-TATA$_{tk}$-CAT reporter, SMRT alone strongly inhibits it, but when present together, one coregulator neutralizes the effects of the other.

DISCUSSION

Antagonist-Mediated Transcriptional Inhibition

Steroid receptor antagonists are pharmacological agents that have been synthesized and selected for clinical use on the basis of empirical tests that show that they inhibit the actions of the cognate natural hormonal agonists. Although there is speculation about the existence of natural antagonists, none have yet been described. The mechanisms by which synthetic antagonists inhibit transcription of steroid hormone-regulated genes have been intensively studied. Antagonists compete with agonists for binding to the HBD of the receptors and then prevent or modify receptor dimerization or DNA binding, or facilitate receptor-DNA interactions but form a transcriptionally nonproductive complex (1, 24, 41, 42). These scenarios confer a passive role on the inhibitory effects of

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

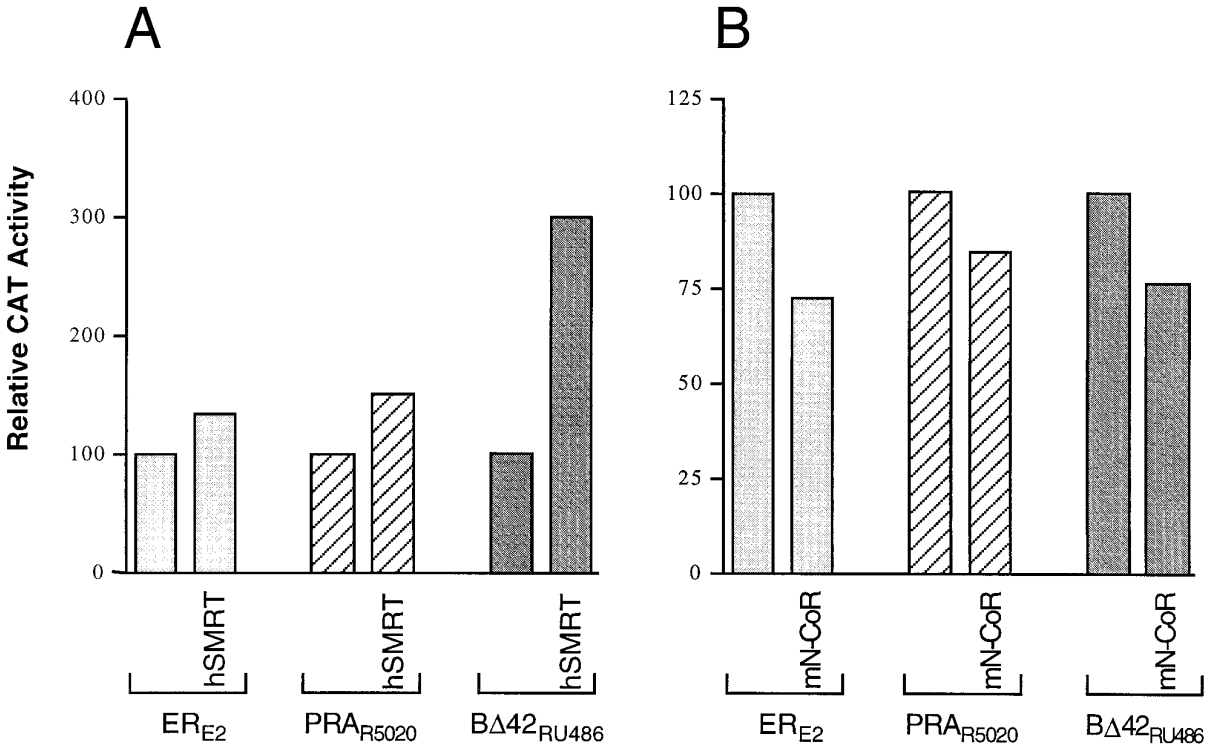

Fig. 9. The Effects of mN-CoR and hSMRT on Agonist-Mediated Transcription

COS cells were cotransfected with 2 μg PRE$_2$-TATA$_{tk}$-CAT or ERE$_2$-TATA$_{tk}$-CAT reporters, 10 ng hER, hPRA, or hPRBΔ42 expression vectors, along with 5 μg hSMRT (A) or mN-CoR (B) expression vectors as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and cells were either untreated or treated with 10 nM of the hormones shown. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantitated by phosphorimaging. *Bars* represent the average of duplicate experimental points. Transcription in the absence of corepressors was set at 100%.

antagonists, in which they simply compete for agonist binding, and therefore block agonist actions.

However, the data presented herein suggest that steroid antagonists can also actively repress transcription. We show that antagonists are able to do so, by recruiting to DNA-bound steroid receptors one or more endogenous corepressors whose normal cellular function is to mediate gene repression by unrelated transcriptional repressors, such as the unliganded retinoic acid/thyroid hormone receptors. We speculate that these corepressors have no normal function with respect to agonist action, but that they are adventitiously brought to promoter-bound steroid receptors, when they are occupied by synthetic antagonists. McDonnell *et al.* (8) and others (48–50) have proposed that agonists and antagonists stabilize different conformational states of steroid receptors. If so, it is possible that a subset of synthetic antagonists freeze DNA-bound steroid receptors in a unique conformational state that enhances the binding affinity of corepressors for the HBD of the receptors. Furthermore, it is possible that on promoters in which steroid receptors repress transcription (16), recruitment of corepressors comes into play.

We have taken advantage of the partial agonist property of RU486 and tamoxifen to demonstrate the recruitment of corepressors to steroid receptors and the resultant transcriptional inhibition. On the other hand, steroids such as ZK98299 (Fig. 1) and ICI 164,384 are also competent antagonists, yet our data suggest that they do not promote receptor-corepressor interactions. It is possible that two, quite different, mechanisms are involved in antagonist-dependent transcriptional inhibition: type I inhibitors may function passively by sequestering the receptors away from the transcription complex, whereas type II inhibitors, which foster receptor binding to DNA, function actively by recruiting corepressors that block transcription. However, no definitive mechanism can be advanced until the controversy surrounding the DNA binding properties, or lack thereof, of antagonist-occupied receptors is resolved.

Recruitment of corepressors explains another physiological puzzle, *i.e*. the ability of some steroid antagonists to suppress gene transcription even in the absence of a hormonal agonist (51). As described above, current assumptions hold that antagonist-occupied receptors suppress agonist-regulated transcription by competitive inhibition. Our model predicts that a gene that contains a steroid hormone response element, but is up-regulated by any signal, including a nonsteroidal one, can be suppressed by recruitment of a corepressor to antagonist-occupied complexes

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

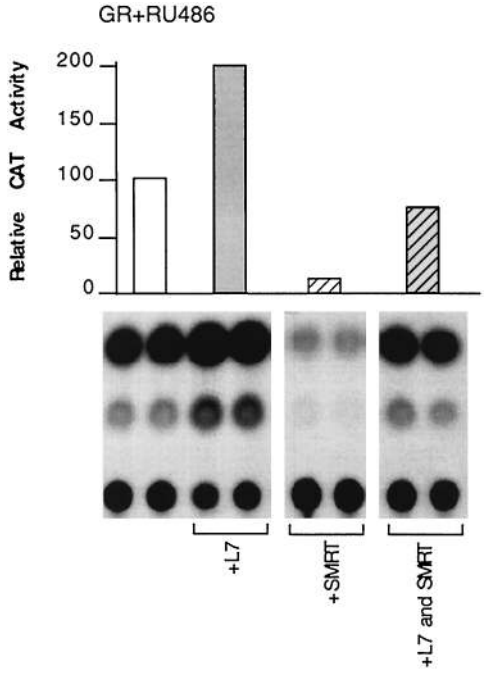

Fig. 10. The Partial Agonist Activity of RU486-Occupied hGR Is Controlled by the Ratio of L7/SPA to SMRT Recruited to the Transcription Complex

COS cells were cotransfected with 2 μg PRE_2-$TATA_{tk}$-CAT reporter, with 10 ng hGR, and with 5 μg L7/SPA or hSMRT expression vectors alone or in combination as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were treated with 100 nM RU486 for 24 h. Cell lysates were normalized to β-galactosidase activity, CAT assays were performed by TLC, and duplicate experimental points were quantitated by phosphorimaging and averaged. The partial agonist activity of RU486-occupied hGR was set at 100% (*open bar*).

bound to the hormone response element of that gene, leading to inhibition in the absence of an agonist.

Partial Agonists and the Coactivator, L7/SPA

When steroid antagonists are used therapeutically, two problems commonly arise. The first is that the drug may have the desired effect in one tissue, but the opposite effect in another. Tamoxifen is a case in point. It is appropriately antiestrogenic in the breast but acts like an estrogen in the uterus, where it induces endometrial cancers (52–56). The mechanisms underlying these undesirable tissue-specific agonist effects are unclear. The second problem arises in tamoxifen-responsive breast cancers, which not only acquire resistance to tamoxifen treatment after a period of time, but in which tamoxifen actively switches to an agonist (7). We have speculated that the mechanisms involved in tissue-specific agonist effects of antagonists, and in the acquired resistance of tumors to tamoxifen treatment, are similar and that both are mediated by coactivators recruited to the transcription complex by the antagonists. Note that tamoxifen-resistant tumors often respond to second-line treatment with a pure antiestrogen or other hormone therapies (57–59), underscoring our contention that pure antagonists operate through mechanisms that differ from those of antagonists with partial agonist activity.

We have now isolated a protein, L7/SPA (29–36), that distinguishes between these two classes of steroid antagonists. In the cytoplasm, L7/SPA associates with the large ribosomal subunit (30), where it inhibits cell-free translation (33). Like other ribosomal proteins, it is a potent autoantigen (32, 34). However, L7/SPA is also an extranucleolar nuclear protein of unknown function (34). Recently an α-helical leucine zipper domain (bZIP) was mapped to the N-terminal 15–49 amino acids of the 248-amino acid protein, through which it homodimerizes and binds to DNA and RNA (31, 35).

We isolated L7/SPA by its ability to bind the H-HBD of hPR, mapped that binding to the hPR hinge region, and showed that L7/SPA strongly enhances transcription by antagonist-occupied hGR, hER, and hPR, but interestingly, that it has no effect on agonist-mediated transcription. L7/SPA therefore exhibits the novel property of being an antagonist-specific transcriptional coactivator whose binding maps to the hinge region. This is the first description, to our knowledge, of an activation function in this region, although an inhibitory function has previously been described (60). Moreover, YL8A (36), the *Saccharomyces cerevisiae* homolog of mammalian L7/SPA, lacks the canonical N-terminal bZIP domain, but the remainder of the molecule shares 56% amino acid identity and 81% conservation with the human protein. As we show in Fig. 1A, RU486 has no partial agonist activity in yeast, suggesting perhaps that the bZIP domain of L7/SPA is important for its coactivator activity, and studies to address this hypothesis are in progress.

There are multiple examples, particularly with tamoxifen, demonstrating agonist activity of antagonists. Several groups have shown that tamoxifen agonism is especially strong on unusual EREs, including the raloxifene response element (61), AP-1 sites (62), and cooperating weak EREs (63). It is interesting to speculate that L7/SPA might be a very potent coactivator at such elements.

Steroid Antagonists and the Combined Effects of Coregulators

The present studies show that antagonist-occupied steroid receptors are targets for the actions of both corepressors and coactivators. It seems logical to suppose that the sum of the combined effects of these coregulatory proteins, determined by their relative cellular concentrations and binding affinities for the receptors, will control the direction of transcription by a particular ligand. This model predicts that the inhibitory or stimulatory efficacy of an antagonist will vary among tissues and tumors depending on the levels

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

and availability of the endogenous coregulators, and suggests that by modulating those levels, it may be possible to control the direction of transcription by the antagonist. Moreover, if the ability to bind an antagonist-specific coactivator is the mark of an antagonist having partial agonist activity, this property should be useful for the pharmacological screening of candidate ligands.

MATERIALS AND METHODS

Plasmid Construction

The hPR H-HBD and hinge region (H or hge) alone, including the complete endogenous nuclear localization signal (NLS), were amplified by PCR and cloned in-frame into the 5′ *Eco*RI and 3′ *Bam*HI sites of the pBTM116 (64) bait plasmid (a gift of Stan Hollenberg, Oregon Health Sciences University, was constructed by Stanley Fields, University of Washington, Seattle, WA and Paul Bartel). The resulting vectors, pLEXA:H-HBD and pLEXA:hge were used in yeast two-hybrid experiments. A third yeast two-hybrid bait vector (pLEXA:HBD) encoding only the HBD of hPR was also constructed by PCR amplification of the HBD as described above and insertion of this fragment into a modified pBTM116 containing the hPR NLS inserted in-frame into the *Pst*I site. A vector encoding the GST fusion protein GST-H-HBD was generated by PCR amplification of the hinge and hormone binding domains of hPR including the entire NLS using primers containing 5′ *Eco*RI and 3′ *Bam*HI sites which were cloned in frame into pGEX 4T1 (Pharmacia, Piscataway, NJ) cut with *Eco*RI and *Bam*HI. The pCMX:mN-CoR construct was a gift from Andreas Hörlein and M. G. Rosenfeld, University of California, San Diego. Full-length L7/SPA was PCR amplified from reverse transcribed HeLa cell cDNA and cloned into the 5′ *Eco*RI and 3′ *Bam*HI sites of the mammalian expression vector pSG5 to create pSG5:L7/SPA. The construct, pLEXA:hN-CoR ID, was made by PCR amplification from the yeast HeLa cell library hN-CoR ID clone of the regions indicated in Fig. 5 and cloned into the 5′ *Eco*RI and 3′ *Bam*HI sites of pBTM116. The hPR1Δ42 mutant was constructed by PCR amplification of a *Hin*dIII-*Bgl*II fragment in the hPR HBD located between amino acids 810 and 891, which was inserted into *Hin*dIII and *Bgl*II-cut hPR1. Wild type hPR and hER expression vectors were obtained from Pierre Chambon (Strasbourg, France), and the hGR expression vector was from John Cidlowski (NIEHS, Research Triangle Park, NC); pCMX-SMRT was a gift from Ron Evans (The Salk Institute, La Jolla, CA). The reporters, PRE_2-$TATA_{tk}$-CAT used for hPR and hGR and ERE_2-$TATA_{tk}$-CAT used for hER, were previously described (65).

Yeast Two-Hybrid System

The plasmid pLEXA:H-HBD was transformed into the yeast two-hybrid reporter strain L40 (64) (MATa his3Δ200 trp1–901 leu2–3, 112 ade2 LYS2::$(lexAop)_4$-HIS3 URA3::$(lexAop)_8$-lacZ GAL4 gal80), a gift from S. Hollenberg, yielding a strain called L40-LEXA:H-HBD. This strain was transfected with a HeLa cell cDNA fusion library cloned into the GAL4 activation domain (AD) vector pGADGH (Clontech, Palo Alto, CA) and plated on appropriate selective media containing 10 μM of the antiprogestin RU486 (Roussel-Uclaf, Romainville, France). Ten million primary transformants were screened for two-hybrid interactions and were detected by growth on histidine drop-out plates and confirmed by β-galactosidase assay. The large-scale library transformation protocol was supplied by Stan Hollenberg and is a modification of published methods (66, 67). Modifications include an overnight growth in liquid media before the histidine selection is applied and the addition of 10 μM RU486 to all growth steps in the transformation protocol.

Yeast β-Galactosidase Assay

Colonies were lifted from original library transformation plates with nitrocellulose filters. Filters were immersed in liquid nitrogen for 15 sec to lyse cells and then placed in petri dishes containing Whatman filters soaked in Buffer ZX (60 mM Na_2HPO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.4 mg/ml X-gal, pH 7.0). Reactions were carried out at 30 C for 8 h.

False-Positive Tests

pLEXA:lamin (64) (a gift of Paul Bartel and Stan Fields) was used to test for nonspecific interactions. The positive GAL4 AD library clones were tested for autonomous activation of reporter genes by β-galactosidase assay and by growth on histidine drop-out media in L40. The GST fusion protein GST-H-HBD was expressed and purified according to published methods (68, 69). The hN-CoR ID and L7/SPA proteins were synthesized and labeled *in vitro* (70, 71). Labeled hNCo-R ID and L7/SPA were incubated with purified GST-H-HBD and glutathione Sepharose 4B matrix, pelleted, and extracted, and protein binding was assessed by SDS-PAGE and autoradiography.

Shuttling Positive GAL AD Clones into *E. coli*

A single positive yeast colony was swirled into ice-cold electro-competent HB101 *E. coli* in a 2-cm electroporation cuvette (Bio-Rad Labs, Hercules, CA). Conditions for electroporation were a pulse at 1500 V, 100 W, and 25 milliFarads (mF) followed by a second pulse 30 sec later at 2500 V, 200 W, and 25 mF in a Gene Pulser (Bio-Rad). Bacteria were plated on M9 media lacking leucine.

Cloning of hN-CoR

To obtain full-length hN-CoR, a human HeLa cell 5′ Stretch Plus cDNA Library (Clonetech, Palo Alto, CA) was screened using two probes. The first probe was the original hN-CoR ID isolated by the two-yeast hybrid screen. The second probe was an N-terminal 1540-bp fragment obtained by RT-PCR from HeLa cell total RNA Template, a sense primer based on the mN-CoR sequence (Genbank MMMU35312) beginning at nt 117 (the translation start site), and an antisense primer designed from a human expressed tagged sequence cDNA (Genbank accession N33258, Genome Systems, St. Louis, MO) corresponding to nt 1540 of the mN-CoR. From this screen two N-terminal clones of approximately 2 kb were obtained that contained 350 bp of 5′-untranslated region, and one 1.8-kb C-terminal clone was obtained that started at nt 6890 in the corresponding mN-CoR sequences and contained approximately 1330 bp of 3′-UT. In addition, by RT-PCR of both HeLa and T47D cell total RNA, a 5564-bp fragment was obtained using the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis, IN), and MuLV reverse transcriptase (Perkin Elmer, Branchburg, NJ), using a sense primer beginning at the translation initiation codon of hN-CoR and an antisense primer initiating within the hN-CoR ID obtained from the yeast two-hybrid clone, and corresponding to nt 5564 of mN-CoR. All of the above fragments were sequenced either manually using Sequenase Version 2.0 (Amersham, Cleveland, OH) or with an ABI 377 sequencer (University of Colorado Health Science Center, Cancer Center DNA Sequencing and Analysis Core). The sequence of hN-CoR was assembled using AssemblyLIGN

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

sequence assembly software (Eastman Kodak Company, Rochester, NY), and compared with mN-CoR using MacVector (Kodak Scientific Imaging, New Haven, CT).

Cell Culture and Transfections

HeLa or COS cells were plated in 100-mm dishes in MEM supplemented with twice charcoal-stripped FCS. Cells were cotransfected by calcium phosphate precipitation (6) with 2 μg reporter plasmid, expression vector (amounts indicated in figure legends), and 3 μg β-galactosidase expression vector pHC110 (Pharmacia-LKB Biotechnology) to normalize for transfection efficiency and carrier DNA for a total of 15 μg/plate. Twenty-four hours after transfection, the cell medium was changed and ligands were added. The following ligand concentrations were used throughout: 100 nM synthetic antagonists RU486 (Roussel Uclaf), ZK98299, ZK112993 (Schering Corp., Berlin, Germany), tamoxifen or ICI164,384 (ICI Pharmaceuticals, Mecclesfield, England) and 10 nM concentrations of the agonists R5020, 17β-estradiol, or dexamethasone. Cells were treated with ligand for 24 h and then harvested. Cell lysates were normalized to β-galactosidase activity, then assayed for chloramphenicol acetyl transferase (CAT) activity by TLC and quantified by phosphorimaging and autoradiography.

Acknowledgments

Received February 5, 1997. Revision received and accepted March 13, 1997.

Address requests for reprints to: Dr. Kathryn Horwitz, Department of Medicine, Division of Endocrinology, University of Colorado Health Center, 4200 East Ninth Avenue, Medicine, Box B151, Denver, Colorado 80262.

We thank S. Hollenberg, R. Evans, M. Rosenfeld, J. Cidlowski, and P. Chambon for reagents; J. Jaehning and R. Sclafani and members of their laboratories for useful discussions and yeast reagents; D. Graham and C. Clarke for the mouse tissue RNA blot; and Roger Powell for expert technical assistance.

Supported by NIH Grants CA-2686G and DK-48238, by Grant DAMD 17–94-J-4391 from the U.S. Army, and by a graduate student stipend from the Lucille P. Markey Charitable Trust (to T.A.J.).

Dedicated to "Grandpa" Bert O'Malley on the happy occasion of his 60th birthday ☺ and to the memory of his first Fellow, William L. McGuire, my teacher and mentor — K.B.H.

Effects of hN-CoR ID and L7/SPA with steroid antagonists were reported at the Steroid/Thyroid/Retinoic Acid Gene Family Keystone Meeting, Lake Tahoe, CA, March 17–23, 1996.

REFERENCES

1. Horwitz KB 1992 The molecular biology of RU486. Is there a role for antiprogestins in the treatment of breast cancer? Endocr Rev 13:146–163
2. Jordan VC 1995 Studies on the estrogen receptor in breast cancer. Breast Cancer Res Treat 36:267–285
3. Nicholson RI, Francis AB, McClelland RA, Manning DL, Gee JMW 1994 Pure anti-oestrogens (ICI164384 and ICI182780) and breast cancer: is the attainment of complete oestrogen withdrawal worthwhile? Endocrine–Related Cancer 1: 5–7
4. Fujimoto N, Katzenellenbogen BS 1994 Alterations in the agonist/antagonist balance of antiestrogens by activation of protein kinalse A signaling pathways in breast cancer cells: antiestrogens selectivity and promoter dependence. Mol Endocrinol 8:296–304
5. Meyer ME, Pomon A, Ji J, Bocquel M-T, Chambon P, Gronemeyer H 1990 Agonistic and antagonist activities of RU486 on the functions of the human progesterone receptor. EMBO J 9:3923–3932
6. Sartorius CA, Tung L, Takimoto GS, Horwitz KB 1993 Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. J Biol Chem 5:9262–9266
7. Horwitz KB 1995 Editorial: When tamoxifen turns bad. Endocrinology 136:821–823
8. McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW 1995 Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. Mol Endocrinol 9:659–669
9. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
10. Mangelsdorf DJ, Evans RM 1995 The RXR heterodimers and orphan receptors. Cell 83:841–850
11. Tsai M-J, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
12. Sap J, Munoz J, Schmitt H, Stunnenberg H, Vennstrom B 1989 Repression of transcription mediated by a thyroid hormone response element by the v-Erb A oncogene product. Nature 340:242–244
13. Damm K, Thompson CC, Evans RM 1989 Protein encoded by v-Erb A functions as a thyroid hormone receptor antagonist. Nature 339:593–597
14. Baniahmad A, Leng X, Burris TP, Tsai SY, Tsai M-J, O'Malley BW 1995 The t4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol Cell Biol 15:76–86
15. Smith DF, Toft DO 1993 Steroid receptors and their associated proteins. Mol Endocrinol 7:4–11
16. Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L 1996 Nuclear receptor coactivators and corepressors. Mol Endocrinol 10:1167–1177
17. Hörlein AJ, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Söderström M, Glass CK, Rosenfeld MG 1995 Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–403
18. Kurokawa R, Söderström M, Hörlein A, Halachmi S, Brown M, Rosenfeld MG, Glass CK 1995 Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature 377:451–454
19. Chen JD, Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
20. Tong G-X, Jeyakumar M, Tanen MR, Bagchi MK 1996 Transcriptional silencing by unliganded thyroid hormone receptor β requires a soluble corepressor that interacts with the ligand-binding domain of the receptor. Mol Cell Biol 16:1909–1920
21. Seol W, Choi H-S, Moore DD 1995 Isolation of proteins that interact specifically with the retinoic X receptor: two novel orphan receptors. Mol Endocrinol 9:72–85
22. Lee JW, Choi H-S, Gyuris J, Brent R, Moore DD 1995 Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. Mol Endocrinol 9:243–254
23. Bocquel M-T, Kumar V, Stricker C, Chambon P, Gronemeyer H 1989 The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell specific. Nucleic Acids Res 17:2581–2595
24. Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS 1996 Tripartite steroid hormone receptor pharmacol-

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

ogy: interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. Mol Endocrinol 10:119–131
25. Beck CA, Weigel NL, Moyer ML, Nordeen SK, Edwards DP 1993 The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. Proc Natl Acad Sci USA 90:4441–4445
26. Sartorius CA, Groshong SD, Miller LA, Powell RP, Tung L, Takimoto GS, Horwitz KB 1994 New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP. Cancer Res 54:3868–3877
27. Fields S, Song OK 1989 A novel genetic system to detect protein-protein interactions. Nature 340:245–246
28. Chien C-T, Bartel PL, Sternglanz R, Fields S 1991 The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA 88:9578–9582
29. Lin A, Chan Y-L, McNally J, Peleg D, Meyuhas O, Wool IG 1987 The primary structure of rat ribosomal protein L7. J Biol Chem 262:12665–12671
30. Tsurugi K, Collatz E, Wool IG, Lin A 1976 Isolation of eukaryotic ribosomal proteins. J Biol Chem 251:7940–7946
31. Witte S, Neumann F, Krawinkel U, Przybylski M 1996 Mass spectrometric identification of leucine zipper-like homodimer complexes of the autoantigen L7. J Biol Chem 271:18171–18175
32. von Mikecz AH, Hemmerich PH, Peter HH, Krawinkel U 1995 Autoantigenic epitopes on eukaryotic L7. Clin Exp Immunol 100:205–213
33. Neumann F, Hemmerich P, von Mikecz A, Peter HH, Krawinkel U 1995 Human ribosomal protein L7 inhibits cell-free translation in reticulocyte lysates and affects the expression of nuclear proteins upon stable transfection into Jurkat T-lymphoma cells. Nucleic Acids Res 23:195–202
34. von Mikecz A, Hemmerich P, Peter HH, Krawinkel U 1994 Characterization of eukaryotic protein L7 as a novel autoantigen which frequently elicits an immune response in patients suffering from systemic autoimmune disease. Immunobiology 192:137–54
35. Hemmerich P, von Mikecz A, Neumann F, Sozeri O, Wolff-Vorbeck G, Zoebelein R, Krawinkel U 1993 Structural and functional properties of ribosomal protein L7 from humans and rodents. Nucleic Acids Res 21:223–31
36. Mizuta K, Hashimoto T, Otaka E 1992 Yeast ribosomal proteins: XIII. *Saccharomyces cerevisiae* YL8A gene, interrupted with two introns, encodes a homolog of mammalian L7. Nucleic Acids Res 20:1011–1016
37. Misrahi M, Atger M, d'Auriol L, Loosfelt H, Meriel C, Fridlansky F, Guiochon-Mantel A, Galibert F, Milgrom E 1987 Complete amino acid sequence of the human progesterone receptor deduced from cloned DNA. Biochem Biophys Res Commun 143:740–748
38. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P 1990 Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9:1603–1614
39. O'Shea EK, Klemm JD, Kim PS, Alber T 1991 X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. Science 254:539–544
40. Turner R, Tjian R 1989 Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. Science 243:1689–1694
41. Bocquel M, Ji J, Ylikomi T, Benhamou B, Vergezac A, Chambon P, Gronemeyer H 1993 Type II antagonists impair the DNA binding of steroid hormone receptors without affecting dimerization. J Steroid Biochem Mol Biol 45:205–215
42. Klein-Hitpass L, Cato ACB, Henderson D, Ryffel GV 1991 Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells. Nucleic Acids Res 19:1227–1234
43. Tung L, Mohamed KM, Hoeffler JP, Takimoto GS, Horwitz KB 1993 Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements, and are dominantly inhibited by A-receptors. Mol Endocrinol 7:1256–1265
44. Umesono K, Evans RM 1989 Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 57:1139–1146
45. Danielsen M, Hinck L, Ringold GM 1989 Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell 57:1131–1138
46. Ptashne M 1988 How eukaryotic transcriptional activators work. Nature 335:683–689
47. Takimoto GS, Tasset DM, Eppert AC, Horwitz KB 1992 Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. Proc Natl Acad Sci USA 89:3050–3054
48. Vegeto E, Allan GF, Schrader WT, Tsai M-J, McDonnell DP, O'Malley BW 1992 The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. Cell 69:703–713
49. Allan GF, Leng XH, Tsai SY, Weigel NL, Edwards DP, Tsai M-J, O'Malley BW 1992 Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. J Biol Chem 267:19513–19520
50. Allan GF, Tsai SY, Tsai M-J, O'Malley BW 1992 Ligand-dependent conformational changes in the progesterone receptor are necessary for events that follow DNA binding. Proc Natl Acad Sci USA 89:11750–11754
51. Thomas M, Monet J-D 1992 Combined effects of RU486 and tamoxifen on the growth and cell cycle phases of the MCF-7 cell line. J Clin Endocrinol Metab 75:865–870
52. Clarke CL, Sutherland RL 1990 Progestin regulation of cellular proliferation. Endocr Rev 11:266–302
53. Curtis RE, Boice J, JD, Shriner DA, Hankey BF, Fraumeni J, JF 1996 Second cancers after adjuvant tamoxifen therapy for breast cancer. J Natl Cancer Inst 88:832
54. Rutqvist LE, Johansson H, Signomklao T, Johansson U, Fornander T, Wilking N 1995 Adjuvant tamoxifen therapy for early stage breast cancer and secondary primary malignancies. J Natl Cancer Inst 87:645–651
55. Kedar RP, Bourne TH, Powles TJ, Collins WP, Ashley SE, Cosgrove DO, Campbell S 1994 Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 343:1318–1321
56. Epstein S, Newcomb PA, Jordan VC, Corbone PP, DeMets DL 1992 Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326:852–856
57. Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME, Clarke R 1993 MCF-7/LCC2: a 4-hydroxy-tamoxifen resistant human breast cancer variant which retains sensitivity to the steroidal antiestrogen ICI182780. Cancer Res 53:3229–3232
58. Lykkesfeldt AE, Sorensen EK 1992 Effect of estrogen and anti-oestrogens on cell proliferation and synthesis of secreted proteins in the human breast cancer cell line MCF-7and a tamoxifen resistant variant subline, AL-1. Acta Oncol 31:131–138
59. Lykkesfeldt AE, Madsen MW, Briand P 1994 Altered expression of estrogen-regulated genes in a tamoxifen-resistant and ICI164384 and ICI182780 sensitive human breast cancer cell line, MCF-7/TAM^{R-I}. Cancer Res 54:1587–1595

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022

60. Adler S, Waterman ML, He X, Rosenfeld MG 1988 Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. Cell 52:685–695
61. Yang NN, Venugopalan M, Hardikar S, Glasebrook A 1996 Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. Science 273:1222–1225
62. Webb P, Lopez GN, Uht RM, Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9:443–456
63. Norris JD, Fan D, Wagner BL, McDonnell DP 1996 Identification of the sequences within the human complement 3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. Mol Endocrinol 10:1605–1616
64. Vojtek AB, Hollenberg SM, Cooper JA 1993 Mammalian Ras interacts directly with the serine/threonine Kinase Raf. Cell 74:205–214
65. Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS, Horwitz KB 1994 A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol Endocrinol 8:1347–1360
66. Gietz RD, Schiestl RH 1989 Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast 7:253–263
67. Schiestl RH, Gietz RD 1989 High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet 16:339–346
68. Smith DB 1993 Purification of glutathione-S-transferase fusion proteins. Methods Mol Cell Biol 4:220–229
69. Frangioni JV, Neel BG 1993 Solubilization and purification of enzymatically active glutathione-S-transferase (pGEX) fusion proteins. Anal Biochem 210:179–187
70. Hope IA, Struhl K 1986 Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell 46:885–894
71. Hope IA, Struhl K 1985 GCN4 protein synthesized *in vitro*, binds HIS3 regulatory sequences: Implications for general control of amino acid biosynthetic genes in yeast. Cell 43:177–188

Downloaded from https://academic.oup.com/mend/article/11/6/693/2754452 by guest on 20 August 2022