Amino-Terminal Domain of TIF2 Is Involved in Competing for Corepressor Binding to Glucocorticoid and Progesterone Receptors†

Dongqing Wang,‡ Qi Wang,§ Smita Awasthi, and S. Stoney Simons, Jr.*

Steroid Hormones Section, NIDDK/CEB, National Institutes of Health, Bethesda, Maryland

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ABSTRACT: Both agonist- and antagonist-bound glucocorticoid receptors (GRs) and progesterone receptors (PRs) regulate gene transcription with the assistance of corepressors (NCoR and SMRT) and coactivators (TIF2/GRIP1, SRC1, and AIB1). Receptor binding of these cofactors is competitive and is considered to involve interactions between the C-terminal ligand binding domain of receptors and receptor interaction domains (RIDs) in the middle and C-terminus of coactivators and corepressors, respectively. Therefore, our recent finding that an amino terminal fragment of TIF2 (TIF2.0 = amino acids 1−627) competed for GR and PR interactions with corepressors in mammalian two-hybrid assays was unexpected. Here, we use biochemical approaches (mammalian two-hybrid, pull-down, and coimmunoprecipitation assays) to locate an N-terminal GR region that is sufficient to bind TIF2.0. In contrast, an N-terminal sequence of PR-B that is largely missing in the shorter PR-A is necessary but not sufficient for TIF2.0 binding. Mutagenesis studies of NCoR establish that the more amino-terminal RID#1, but not RID#2, is necessary for binding to both GR and PR agonist and antagonist complexes. ChIP assays indicate that PR and NCoR each selectively localize to the enhancer element (PRE) of a transiently transfected PREtkLUC reporter in the presence of antagonist steroid, whereas exogenous TIF2.0 reduces the amount of PRE-associated NCoR. Importantly, exogenous TIF2.0 also inhibits the biological responses to added NCoR under the same conditions as those used in the ChIP assays. These results suggest that both N-terminal and middle sequences of TIF2 participate in competing with corepressor for regulating the gene transcriptional responses of GRs and PRs.

The ability of associated factors to modify steroid receptor activities in regulating gene transcription has become a dominant feature in the mechanism of steroid hormone regulated gene transcription (*1*−*4*). After steroid binding to the classical intracellular steroid receptors and conversion of the receptor−steroid complex to the activated state with increased affinity for DNA and nuclei, receptors bind to biologically active hormone response elements (HREs[1]) to initiate changes in the rates of gene expression. This task is facilitated and transmitted by the binding of selected factors to the HRE-bound activated receptor−steroid complexes (*5*, *6*) and the subsequent recruitment of a host of transcriptional cofactors. Two prominent classes of initially binding cofactors are the p160 coactivators (SRC-1, TIF2/GRIP1, and AIB1/pCIP/ACTR/RAC3/TRAM1) and the corepressors NCoR and SMRT. NCoR and SMRT were discovered by virtue of their ability to repress the transcriptional activity of DNA-bound nuclear receptors (*7*, *8*) and were initially thought to be associated with ligand-free or antagonist-bound receptors. Conversely, coactivators were considered to be bound only to agonist-bound receptors.

Recent studies of coactivator and corepressor associations indicate that a less regimented situation exists with the classical steroid receptors. Coactivators and corepressors can reverse the biological effects of each other with both agonist and antagonist complexes of androgen (AR), glucocorticoid (GR), mineralocorticoid, and progesterone receptors (PR) in intact cells (*4*, *6*, *9*−*19*). These biological responses are consistent with both cell-free observations (*4*, *6*, *14*, *15*, *18*, *20*) and whole-cell fluorescence measurements (*17*, *19*) of coactivators and corepressors binding to both receptor−agonist and receptor−antagonist complexes. Thus, by varying the ratio of coactivators to corepressors, and the amounts of other cofactors, it is possible to achieve a continuum of responses, as if tuning the properties of gene expression with a rheostat. Such fine control of steroid hormone action offers tremendous possibilities for the differential expression of steroid-regulated genes within and between cells and tissues. However, before one can capitalize on the potential of coactivators and corepressors as viable therapeutic targets for treating endocrine disorders, a much better understanding of the molecular mechanisms by which these cofactors alter steroid receptor-mediated transcription is required.

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* To whom correspondence should be addressed. Dr. S. Stoney Simons, Jr., Bldg. 10, Room 8N-307B, NIDDK/CEB, NIH, Bethesda, MD 20892-1772. Phone: 301-496-6796. Fax: 301-402-3572. E-mail: steroids@helix.nih.gov.

‡ Current address: Dr. Dongqing Wang, Johns Hopkins Bayview Medical Center, 4940 Eastern Ave., B114, Baltimore, MD 21224.

§ Current address: Dr. Qi Wang, Beth Israel Deaconess Medical Center, HIM Building, Room 1043, 330 Brookline Ave, Boston, MA 02115.

[1] Abbreviations: AR, androgen receptor; co-IP, co-immunoprecipitate; GR, glucocorticoid receptor; HRE, hormone response element; LBD, ligand binding domain; PR, progesterone receptor; PRE, progesterone response element; RID, receptor interaction domain.

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Previous studies revealed that LxxLL motifs of the p160 coactivators (where L is a leucine, and x is any amino acid) are necessary and sufficient for binding to a cleft in the ligand binding domain (LBD) of steroid receptors that is formed by helices 3, 4, 5, and 12 (*21*, *22*). Similarly, corepressors NCoR and SMRT were found to utilize a related, extended CoRNR-box sequence of LxxI/HIxxxI/L to bind to a site in the LBD that overlaps with, but is not identical to, the coactivator binding site (*20*, *23*−*25*). Recently, we found that the ability of TIF2 to compete for corepressor binding to GRs and PRs in mammalian two-hybrid assays was retained by an amino terminal fragment of TIF2 (*6*, *14*), called TIF2.0 (*26*). Unexpectedly, a more C-terminal construct of TIF2 containing the receptor interaction domains (RIDs), which is known to bind to GRs, called TIF2.4 (*26*) did not prevent corepressor binding to GRs or PRs. These results suggest that TIF2.0 binds to some unidentified region of GR and PR and that TIF2.0 contributes to the biological properties of TIF2 with steroid receptors.

Several cofactors are reported to interact with GR and PR domains other than the LBD. GRs lacking the LBD bind both SRC-1 and AIB1 (*27*). Both TSG101 and DRIP150 associate with the N-terminal AF1 domain of GR (*28*). SRA (*29*) and BRCA1 (*30*) combine with the PR N-terminus. The C-terminal region of several p160 coactivators interacts with the N-terminal domains of steroid receptors (*31*−*34*). Protein-induced folding of the normally disordered AF1 domain of GRs and ARs increases the affinity for SRC-1, CBP, and TBP (*35*). The osmolyte TMAO also increases the binding of AF1 with some (CBP, TBP, and SRC-1) but not all (TIF2/GRIP1, NCoR, RIP140, and AIB1) cofactors (*36*). SRC-1 and TIF2 each are reported to increase the AF1 activity of GRs and PRs (*32*), and TIF2 augments the transcriptional activity of PR-B but not the N-terminal truncated PR-A (*37*). However, we are not aware of any description of the amino terminus of TIF2 binding to GR or PR, or of TIF2 binding to any other part of GR or PR other than the LBD.

The purpose of this study was, therefore, to determine whether the competitive biological activity of TIF2.0 with corepressor actions on GRs and PRs involves a direct interaction with receptors. Several biochemical assays with the isolated TIF2.0 fragment indicate that TIF2.0 does bind to related but non-homologous N-terminal domains of GR and PR. The overlap of the TIF2.0 binding region with that required for NCoR binding is much greater for GRs than for PRs. Nevertheless, ChIP assays demonstrate that TIF2.0 competitively inhibits NCoR binding to the enhancer region of an exogenous PR-regulated gene under conditions where TIF2.0 reverses the biological effects of NCoR on PR-mediated gene expression. Thus, we conclude that N-terminal sequences of GR, PR, and TIF2 contribute to the binding and biological actions of coactivators and corepressors with GRs and PRs.

EXPERIMENTAL PROCEDURES

Unless otherwise indicated, all operations were performed at 0 °C.

Chemicals. Dex was obtained from Sigma (St. Louis, MO), and promegestone (R5020) was from PerkinElmer Life Sciences (Boston, MA). RU486 was a gift from Etienne Baulieu (Paris, France). Restriction enzymes and DNA polymerase were from New England Biolabs (Beverly, MA), Amersham Biosciences (Piscataway, NJ), or Promega (Madison, WI). [^{35}S]Methionine was from Amersham Biosciences.

Plasmids. The *Renilla* null luciferase reporter was purchased from Promega (Madison, WI), and pFR-LUC reporter from Stratagene (La Jolla, CA). Renilla-TS reporter was a gift from Drs. Nasreldin M. Ibrahim and Otto Fröhlich (Department of Physiology) and Dr. S. Russ Price (Department of Medicine) at Emory University School of Medicine (Atlanta, GA). pSG5/human serum albumin (hSA), VP16/GR, VP16/GR361C, VP16/GR407C, pRBAL-GR, pRBAL-GRN523, and pRBAL-GR486C have been described previously (*14*). Chimeras of the VP16 activation domain and PR-B, PR-A, PR-BN, and PR-AN (all human) were gifts from Dean P. Edwards (University of Colorado Health Sciences Center, Denver, CO). Other donated plasmids were received from Hinrich Gronemeyer (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France: TIF2, TIF2.0, and hPR-B, hPR-A), Michael Rosenfeld (University of California-San Diego, San Diego, CA: NCoR), Ron Evans (Salk Institute, La Jolla, CA: s-SMRT), Keith Yamamoto (University of California-San Francisco, San Francisco, CA: pSVLGR), Michael R. Stallcup (University of Southern California, Los Angeles, CA: pSG5-HA and HA/GRIP1), and Mitch Lazar (University of Pennsylvania School of Medicine: GAL/NCoR-RID (amino acids 1944−2453), GAL/NCoR-RIDm1, GAL/NCoR-RIDm2, GAL/SMRT-RID (amino acids 982−1495), and GST/NCoR-RID).

The PR and GR chimeras VP16/PR395C, VP16/PR468C, VP16/PR562C, VP16/GR157C, VP16/GR237C, VP16/GR310C, VP16/GR361C, VP16/GR407C, VP16/GR486C, VP16/GR154-236, VP16/GR206-236, and VP16/GR/PR/GR, and PREtkLUC (= GREtkLUC) have been described previously (*6*). The following procedures were used to obtain the other GR constructs for in vitro transcription/translation. For VP16/GR201-523, three fragments were ligated: the 0.6-kb fragment obtained by SmaI/Pst I digestion of VP16/GRN523 and the 0.3-kb fragment from EcoRI /SmaI digestion of the PCR-amplified region of VP16/GR, using the forward primer 5′-TCGGAATTCAGTGTGAAATTG-TATCCC-3′ and the reverse primer 5′-TATGGATCCT-CAAAGGGATGCTGTATTCAT-3′, were ligated with the 3.3-kb fragment from EcoRI /Pst I digestion of VP16. VP16/GR261-523, and VP16/ GR361-523 were prepared in the same manner, with the same reverse primer 5′-TCCTG-CAGTGGCTTGCTGAAT-3′ but using the forward primer 5′-GCCGAATTCACGAATGAGGATTGTAAG-3′ for amino acids 261−523 and the forward primer 5′-GCCGAATTC-CTTTCTCAGCAGCAGGAT-3′ for amino acids 361−523. EcoRI/Pst I digestion of PCR products was ligated with the 3.3-kb fragment from EcoRI/Pst I digestion of VP16. For pBAL-GR201C, the 1.7-kb fragment from BamHI digestion of the PCR-amplified region of pSVLGR using the forward primer 5′-ATGGATCCATGAGTGTGAAATTGTATC-CCAC-3′ and the reverse primer 5′-TATCTAGAGT-CATTTTTGATGAAACAG-3′ was ligated with the 3.0-kb fragment from BamHI/SmaI digestion of pBAL-GR. For pSG5-HA/TIF2.0, the EcoRI and Bgl II digestion product of pSG5-HA was ligated with EcoRI- and Bgl II-treated pSG5-TIF2.0 as described previously (*38*).

Cell Culture and Transfection. Monolayer cultures of Cos-7, CV-1, and 1470.2 cells were grown as described previously (*10*, *39*). Cells are transfected for 18 h using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) or FuGene (Roche Diagnostics, Indianapolis, IN) as recommended by the supplier. For each well of a 24-well plate, we used 100 ng of reporter (FR-LUC or PREtkLUC) and 5 or 10 ng of Renilla-TS plus various combinations of other expression vectors. Equal molar amounts of expression vectors lacking GR, PR, or cofactors (i.e., pCMX-hSA, pSG5-hSA, VP16, or Gal) are included to keep the molar amount of each vector constant, with the total transfected DNA brought to 300 ng/well with pBSK+ unless otherwise indicated. The cells were then treated for 20−24 h with 0.1% ethanol ± steroids in media containing 10% FBS and harvested in 1× Passive Lysis Buffer (150 μL/well; Promega). Cell lysates (50 μL) were used to assay for luciferase activity using the Dual-Luciferase Assay System from Promega according to the supplier's instructions. The data are normalized for *Renilla* activity to correct for differences in transfection efficiency. The fold induction by a steroid is defined as (the activity with steroid)/(the basal level activity seen in the absence of hormone). The partial agonist activity of a steroid A (expressed as percent) is defined as follows: 100 × [(the activity with 1 μM steroid A) − (the basal level seen in the absence of hormone)]/[(the activity with 1 μM Dex) − (the basal level seen in the absence of hormone)]. The amount of each receptor plasmid used is determined from titration experiments to be less than that required for maximal gene induction, thus ensuring that the receptor protein is limiting for each condition. Dose−response curves were generated by KaleidaGraph (Synergy Software, Reading, PA) as the best fit for Michaelis−Menton kinetics (R^2 always ≥0.97).

Bacterial Expression of Proteins. The pGEX series of plasmids were transformed into *Escherichia coli* (BL21 [DE3]; Stratagene) according to the manufacturer's procedure. A single colony was picked and inoculated into 3 mL of Luria−Bertani broth with 100 μg/ml ampicillin. After overnight culture, 1 mL of bacterial culture was diluted into 50 mL of Luria−Bertani broth containing 100 μg/ml ampicillin, shaken at 37 °C for 2 h, adjusted to 0.5 m/ml isopropyl-β-D-thiogalacto-pyranoside, and shaken at 25 °C for another 3 h. The cells were harvested by centrifugation, washed once with PBS, resuspended in 10 mL of PBS, and sonicated for 30 cycles at 30% of maximum power (Fisher Scientific sonic dismembrator, model 500). The supernatant was collected for use after centrifugation (5000*g* for 20 min).

Transcription and Translation Assays in Vitro. For each reaction, 1 μg of plasmid DNA was mixed with 2 μL of [^{35}S]methionine and 40 μL of TNT T7 (or SP6) master mix (Promega) and brought up to a total volume of 50 μL with H_2O. The reaction was conducted at 30 °C for 90 min in the presence of various steroid hormones (Dex, RU486).

Affinity Purification of HA-Tagged Proteins. Cos-7 cells were seeded into 150 mm dishes at approximately 3 × 10^6 cells per dish. The next day, Cos-7 cells were transfected with pSG5-HA/GRIP1 or pSG5-HA/TIF2.0. On the third day, the cells were rinsed once with PBS and ruptured in 2 mL Cytobuster lysis buffer (NOVAGEN). The cells were scraped off the plate, collected, and centrifuged at 13,000*g* for 10 min. The HA-tagged proteins in the supernatant were purified on an anti-HA affinity matrix according to the supplier (Roche).

Pull-Down Assays. Sonicated bacterial lysates (0.5 mL) containing overexpressed GST or GST/NCoR-RID were incubated with 20 μL of glutathione-Sepharose 4B beads for 1 h at 0 °C. The mixture was centrifuged (12,000*g*), the supernatant discarded, and the matrix was washed with PBS (4 × 1 mL). Each 20 μL sample of immobilized HA or HA-chimera was then incubated overnight at 0 °C with 10 μL of hormone-prebound and activated, ^{35}S-labeled in vitro-translated GR. The matrix was washed with 4 × 1 mL of Buffer H (150 mM KCl, 20 mM HEPES (pH 7.8), 2 mM EDTA (pH8.0), 0.1% NP-40, 10% glycerol, 0.5% nonfat dry milk, and 5 mM DTT). The immobilized proteins were removed from the beads by heating at 90 °C for 5 min in 20 μL of 2× sodium dodecyl sulfate (SDS) loading buffer. The proteins were then separated on 8 or 10% SDS−PAGE gels, and the bound receptor was located by autoradiography.

Coimmunoprecipitation Assays. Transiently transfected Cos-7 cells in 150 mm dishes were lysed at room temperature with CytoBuster Protein Extraction Buffer (EMD Biosciences, La Jolla, CA) and clarified by centrifugation at 20,000*g* for 20 min at 4 °C. Complexes with HA-tagged proteins were immobilized on Anti-HA Affinity Matrix (Roche) with rocking (100 cycles/min) at 0 °C overnight and then washed three times with 1 mL of ice-cold wash buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and complete protease inhibitor cocktail (Roche)). The proteins were extracted with 5× SDS sample buffer, separated by SDS−PAGE, and visualized by Western blotting with BUGR2 anti-GR antibody (Affinity BioReagents, Inc., Golden, CO).

Western Blotting. SDS−PAGE gels were equilibrated in transfer buffer for 15 min at room temperature before electrophoretic transfer of the proteins to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH) in a Bio-Rad (Hercules, CA) small (150−200 mA overnight) or large (350 mA overnight) Transblot apparatus. The nitrocellulose membranes were stained with Ponceau S (0.02% Ponceau S and 0.04% glacial acetic acid in water) to localize the molecular weight markers. The VP16 fusion proteins were probed with rabbit VP16 polyclonal antibody (CLONTECH). Antibody complexes were visualized by ECL detection reagents as described by the manufacturer (Amersham Biosciences).

ChIP Assays. The ChIP assays were performed essentially as described (*40*). Two days before harvest, 1470.2 cells were seeded into 150 mm dishes at approximately 3 × 10^6 cells per dish. The next day, plasmids (15 μg/dish) were transfected. On the third day, after appropriate ligand treatment, formaldehyde (37%) was directly added into the medium to a final concentration of 1% for 10 min, and then 1.25 M glycine was added to a final concentration of 0.125 M for 5 min. Cells were washed twice with cold PBS and harvested by scraping into 5 mL of cold PBS containing protease inhibitors. After centrifugation at 1700*g* for 10 min, the pellets were resuspended in nuclear lysis buffer and sonicated by Fisher Scientific Ultrasonic Dismembrator (model 500) and then centrifuged at 13,000*g* for 10 min. The soluble chromatin was precleared with Protein G beads (40 μL) (Amersham Pharmacia) at 0 °C for 5 h, followed by centrifugation. The supernatant containing precleared chromatin was then incubated with antibodies for PR or NCoR

(Affinity BioReagents (PA1-844 and -844A); Santa Cruz Biotechnology (SC-1609)) at 0 °C overnight. Protein G (40 μL) was added, the mixtures were incubated for 2 h at 0 °C, and the immune complexes were collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5−10 min in wash buffers I, II, and III (*40*) and 3× 1 mL of TE. Two 150 μL aliquots of elution buffer were used to obtain the product. The NaCl concentration in the combined eluents (300 μL) was adjusted to 300 mM with 5 M NaCl, and formaldehyde cross-linking was reversed by heating the samples at 65 °C overnight. Proteinase K (2 μL of 10 μg/μL, Sigma) was then added to each sample in buffer (50 mM Tris-HCl (pH 8.5), 1% SDS, and 10 mM EDTA) and incubated for 1 h at 45 °C. The DNA was purified by Qiagen PCR Purification Kit per manufacturer's instructions. The primers used for ChIP analysis are as follows: for PRE, the forward primer was 5′-GTTGTAAAACGACGGCCAG-3′ and the reverse primer 5′-GAGGCCACACGCGTCACCTTAATA-3′; for LucF2, the forward primer was 5′-CCAGGGATTTCAGTC-GATGT-3′ and the reverse primer 5′-AATCTGACGCAG-GCAGTTCT-3′; for Chip3, the forward primer was 5′-ATTCCACACAACATACGAGCC-3′ and the reverse primer 5′-TTATCCCCTGATTCTGTGG-3′; for 4441, the forward primer was 5′-GTCATGAGATTATCAAAAAGG-3′ and the reverse primer 5′-ACTGATTAAGCATTGGTAAC-3′.

qRT-PCR. For the quantitative analysis, real-time quantitative PCR was carried out using the same primers as those used in the ChIP assay. The ABI Prism 7900HT Detection System (Applied Biosystems) was employed using the DNA binding dye SYBER Green (PE Applied Biosystems) for the detection of PCR products. The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced products of the expected size.

Statistical Analysis. Unless otherwise noted, all experiments were performed several times in triplicate. The values of *n* independent experiments were then analyzed for statistical significance by the two-tailed Student's *t*-test using the program InStat 2.03 for Macintosh (GraphPad Software, San Diego, CA). When the difference between the SDs of two populations was significantly different, then the Mann−Whitney test or the Alternate Welch *t*-test was used.

RESULTS

TIF2.0 Interaction with the Corepressor NCoR. The simplest explanation for how TIF2.0 inhibits GR interactions with the corepressor fragment NCoR-RID (*14*) is that TIF2.0 binds to either NCoR or GR to prevent GR-NCoR association. We find that the relatively poorly translated full-length TIF2 and the more efficiently translated HA/GRIP1 both interact with the corepressor in a pull-down assay (arrows in Figure 1). This is consistent with the earlier observation of Li et al. that NCoR binds strongly to the related p160 coactivator AIB1 and weakly to TIF2 in pull-down and two-hybrid assays (*41*). However, TIF2.0 and HA/TIF2.0 do not interact with NCoR-RID under the same conditions. Therefore, coactivator binding to corepressors may participate in TIF2 antagonism of corepressor actions but cannot account

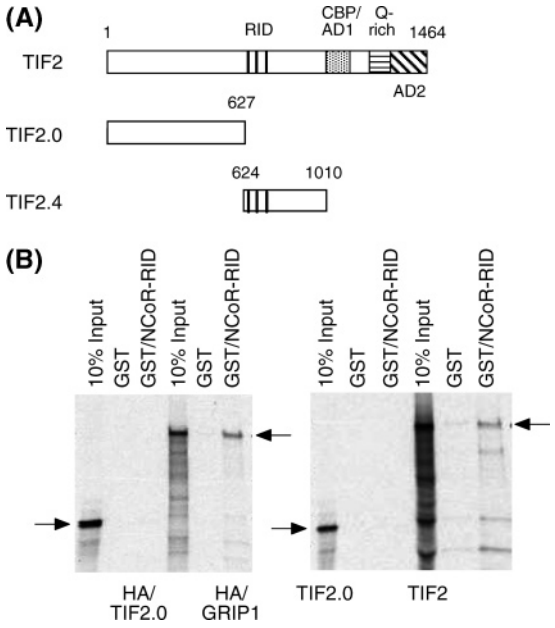

FIGURE 1: Coactivator binding to corepressor. (A) TIF2/GRIP1 constructs used in assays. Cartoons show the various domains present in full length and truncated TIF2 constructs: RIDs (solid bars), activation domain 1 (AD1)/CBP binding domain (stippled), Q-rich (horizontal striping), and AD2 (cross hatched). (B) TIF2/GRIP1 binding to NCoR-RID in cell-free pull-down assay. [^{35}S] in vitro translated full length TIF2/GRIP1 or TIF2.0, ± HA tag, was visualized by autoradiography (at the positions of arrows) after being bound to columns containing immobilized GST or GST/NCoR-RID. Lane labeled 10% input contains 10% of in vitro translated proteins added to each column.

for the inhibition by TIF2.0 of the effects of NCoR or SMRT with GR and PR (*6*, *14*).

TIF2.0 Interaction with GR. Mammalian two-hybrid assays were used to examine the interactions between TIF2.0 fused to the DNA binding domain of GAL4 and full-length GR attached to the activation domain of VP16. We see a strong association between GR and TIF2.0 in CV-1 cells in the presence of both the agonist Dex and the antagonist RU486 (Figure 2A). Subsequent studies were usually performed in Cos-7 cells, which, like CV-1 cells, are also derived from green monkey kidney cells, because PR displays a much stronger interaction with TIF2.0 in Cos-7 than in CV-1 cells (data not shown). In order to identify the region responsible for binding to TIF2.0, we examined the interaction of GAL/TIF2.0 with a series of N-terminally truncated GR chimera in Cos-7 cells. For both Dex- and RU486-bound GRs, the amount of induced luciferase decreased significantly upon deleting the residues between positions 157 and 309 (Figure 2B). Western blots (Figure 2C and ref *14*) confirm that the inactivity of the shorter constructs is not due to lower levels of expressed protein.

These data were supported by pull-down assays in which *in vitro* translated ^{35}S-labeled GR constructs were adsorbed to matrices containing immobilized, HA-tagged TIF2.0 (Figure 2D). For comparison, we looked at HA-tagged GRIP1, the mouse homologue of TIF2. Consistent with the data of Figure 2B, TIF2.0 retention of Dex-bound GR is lost when GR sequences between amino acids 201 and 360 are removed. In contrast, binding of GR−Dex complexes to full-length TIF2/GRIP1 is seen for all GR constructs. No steroid was added with GRN523 because the LBD is absent. We

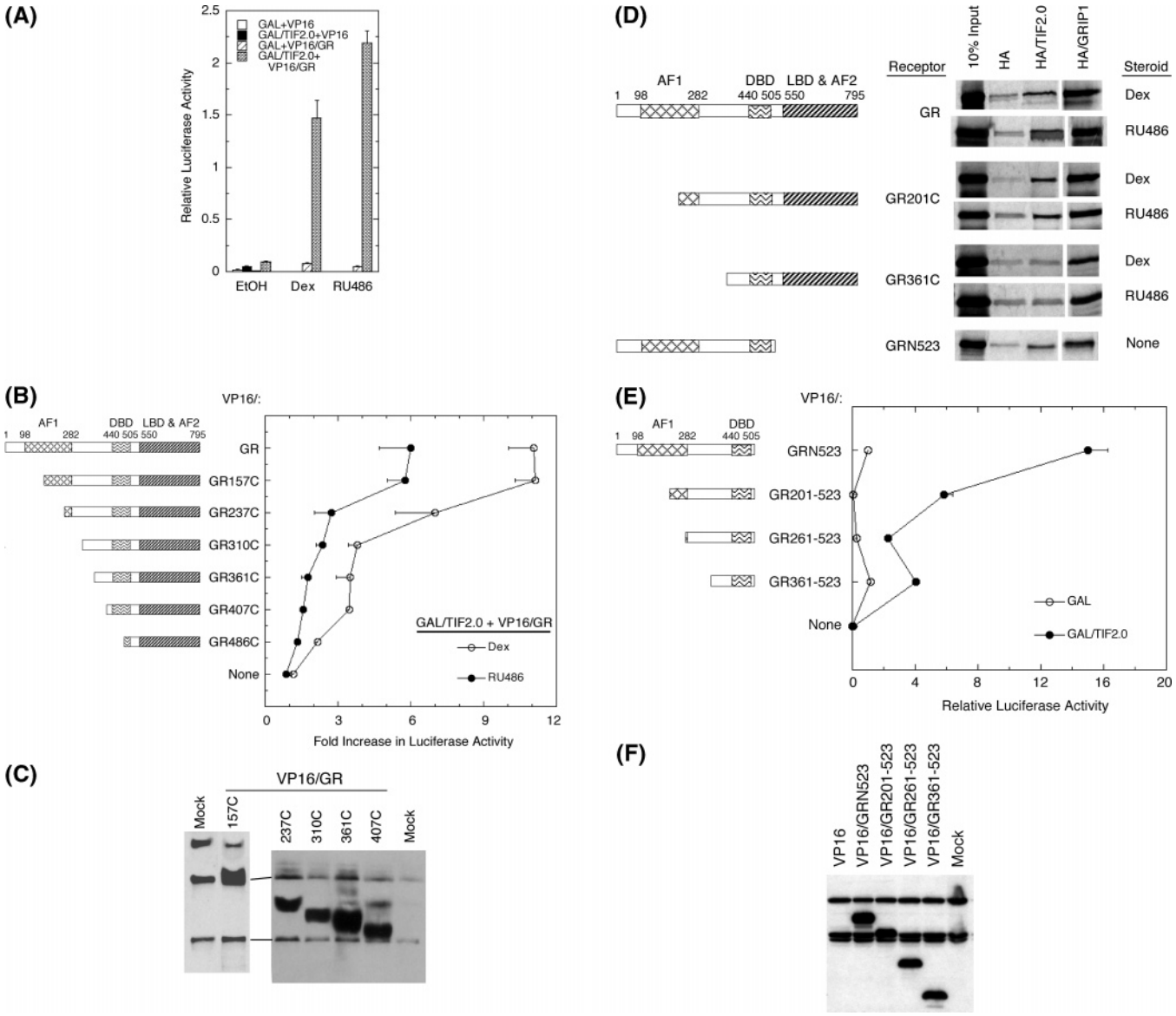

FIGURE 2: GR binding to TIF2.0. (A) Mammalian two-hybrid assay of VP16 ± GR binding to GAL ± TIF2.0. Triplicate wells were composed as indicated and treated with EtOH, 1 μM Dex, or 1 μM RU486 overnight before being analyzed and plotted ± SD as described in Experimental Procedures. Similar results were obtained in two additional experiments. (B) Mammalian two-hybrid assay of GAL/TIF2.0 binding to VP16 ± GR with increasing N-terminal deletions. Cartoons show regions of GR that are retained in each construct, with the numbers above each structure indicating the amino acid position of each domain boundary and the letter C designating the C-terminal residue of the receptor. The assay was conducted as described in A, after which the average values from three experiments were plotted as fold induction of Luciferase activity (± SEM) above basal activity by 1 μM Dex or RU486. (C) Western blot of VP16/GR chimeras of B from transiently transfected Cos-7 cells with anti-VP16 antibody. (D) Binding of GR deletion constructs to TIF2/GRIP1 in cell-free pull-down assays. In vitro translated [^{35}S] GR constructs (see part B) were visualized by autoradiography on a single gel after being bound to columns containing immobilized HA ± TIF2/GRIP1 constructs (see Figure 1A). Lane labeled 10% input contains 10% of in vitro translated proteins added to each column. Cartoons (left) show the constructs used. (E) Mammalian two-hybrid assay of GAL ± TIF2.0 binding to VP16 ± GRN523 (N = amino acid 1) with increasing N-terminal deletions. Cartoons (left) show the constructs used. The assay was conducted as described in A, after which the average values of normalized total activity (VP16/GRN523 with GAL = 1) from two to four experiments were plotted (± SEM). (F) Western blot of VP16/GR chimeras of E from transiently transfected Cos-7 cells with anti-VP16 antibody.

therefore conclude that N-terminal sequences of GR are required for TIF2.0 binding.

To determine whether GR N-terminal sequences are sufficient for TIF2.0 binding, mammalian two-hybrid assays were conducted with a series of N-terminal constructs. As shown in Figure 2E, a strong interaction is seen with VP16/GRN523. Most of this response is lost upon deletion of the N-terminal 261 amino acids, consistent with the data in Figure 2B. The Western blots of Figure 2F indicate that the inactivity of the two shortest constructs of Figure 2E is not due to insufficient levels of protein expression.

Coimmunoprecipitation assays were performed with wild-type GRs and HA-tagged TIF2.0 to further investigate the whole-cell interactions of these species and to rule out artifactual interactions between GAL-DBD and the VP16 activation domain. Immunoprecipitation of HA/TIF2.0 with anti-HA antibody coimmunoprecipitates (co-IPs) both steroid-free and steroid-bound full-length GRs (Figure 3A, lanes 5−7) of VP16/GRN523. GRN523 comigrates with HA/TIF2.0 (data not shown). Therefore, VP16/GRN523 was used to achieve a spatial separation from HA/TIF2.0, with VP16/GR as a control. Ligand-free VP16/GR is co-IP'd with HA/

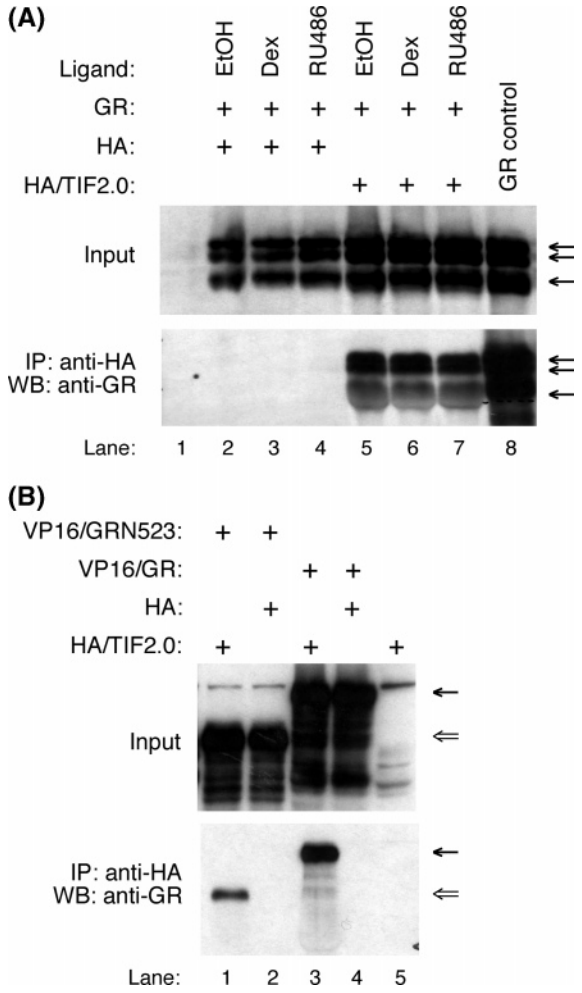

FIGURE 3: Coimmunoprecipitation of GR with TIF2.0. (A) Association of GR with HA/TIF2.0. Cytosols from Cos-7 cells that were transiently transfected with GR and HA ± TIF2.0 and then treated with EtOH ± 1 μM Dex or RU486 were separated by SDS−PAGE either before (Input) or after immunoprecipitation by anti-HA antibody. Coimmunoprecipitated GR (arrows) was visualized by Western blotting with anti-GR antibody. (B) Association of VP16/GRs with HA/TIF2.0. Cos-7 cells were transiently transfected with VP16/GR or VP16/GRN523 and processed as described in A except that no steroid was added. Positions of VP16/GR and VP16/GRN523, detected by anti-GR antibody, are indicated by simple and open arrows, respectively. Endogenous and overexpressed wild type GR, but not VP16/GR chimera, gives smaller, amino-terminal truncated species because of downstream translational start sites (*64*, *65*).

TIF2.0 (Figure 3B, lane 3 vs lane 4), just as seen with wild-type GR in Figure 3A. At the same time, VP16/GRN523 also co-IPs with HA/TIF2.0 in a manner that requires the presence of TIF2.0 (lane 1 vs lane 2 of Figure 3B). Collectively, these data argue that the N-terminal half of GR is necessary and sufficient for binding to TIF2.0 in a steroid-independent manner, with the large segment of amino acids 157−261 being essential.

TIF2.0 Interaction with PR. Coactivators and corepressors antagonize the effects of each other with PRs (*12*), and TIF2.0 also inhibits PR interactions with NCoR (*6*). We therefore used the assay system of Figure 2A to examine the ability of VP16/PR constructs to interact with GAL/TIF2.0. As for GRs, a steroid-induced increase in association with TIF2.0 depends upon amino terminal sequences of PR that are largely missing in the shorter PR-A isoform

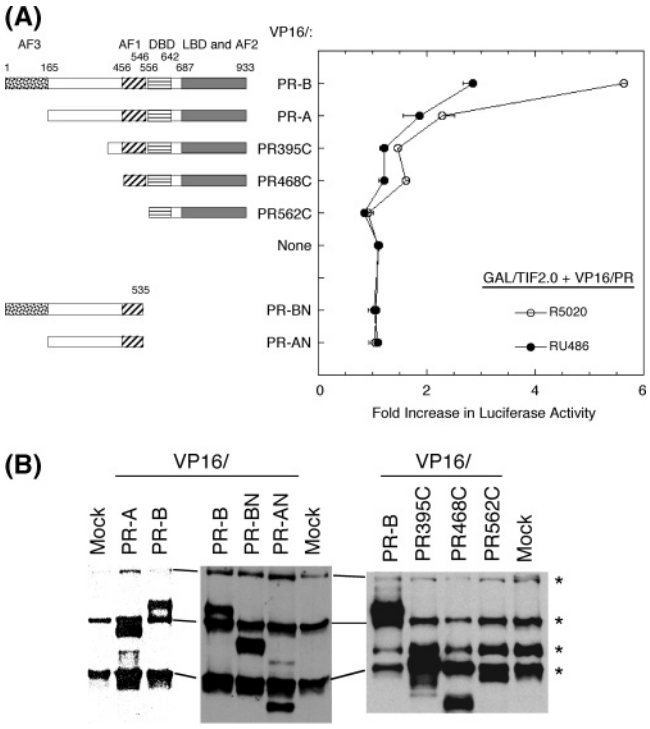

FIGURE 4: PR binding to TIF2.0. (A) Mammalian two-hybrid assay of GAL/TIF2.0 binding to VP16 ± various deletions of PR. Cartoons (left) show constructs used. Triplicate assays were performed, and the average value from three experiments (± SEM) were plotted, as in Figure 2B, as the fold induction by 20 nM R5020 or 1 μM RU486 with the indicated VP16/PR constructs. (B) Western blot of VP16/PR chimeras of A from transiently transfected Cos-7 cells with anti-VP16 antibody. Asterisks (*) indicate the nonspecifically detected species.

(Figure 4A). However, in contrast to GRs where the N-terminal half is sufficient, the PR segment of amino acids 1−535 is unable to productively interact with TIF2.0, even though it contains the residues (1−395) that are required for TIF2.0 association. Figure 4B (and ref *6*) show that the inactivity of the truncated PR constructs is not due to low levels of protein expression.

Regions of GR and PR N-Terminal Domains Required for Interaction with Corepressors. Having identified the regions of GR and PR that are necessary for association with TIF2.0, we needed to define the area involved in corepressor binding in order to test our hypothesis that TIF2.0 and corepressors competitively inhibited the binding of each other to receptors. The data of Figure 5A reveal that the GR segment between amino acids 157 and 361 is responsible for most of the interaction between GR and NCoR-RID. Almost identical results are obtained for GR and SMRT-RID (Figure 5B), which is a C-terminal segment of SMRT that contains the RIDs and is sufficient for binding to GRs (*6*, *14*, *20*, *42*). CV-1 cells were used in this experiment because the two-hybrid interactions of GR are stronger in CV-1 than in Cos-7 (data not shown) or 1470.2 cells (*6*, *14*), thus permitting a better resolution of the effects of GR deletions. The pattern of binding to SMRT is very similar to that for both NCoR and TIF2.0 binding to GR in Figures 5A and 2B, respectively. The region needed for corepressor binding is somewhat larger than that for TIF2.0 binding, but both are encompassed by amino acids 157−406. The major difference is that the interactions with corepressors are greatest for GR-bound by the antagonist RU486, whereas the agonist Dex

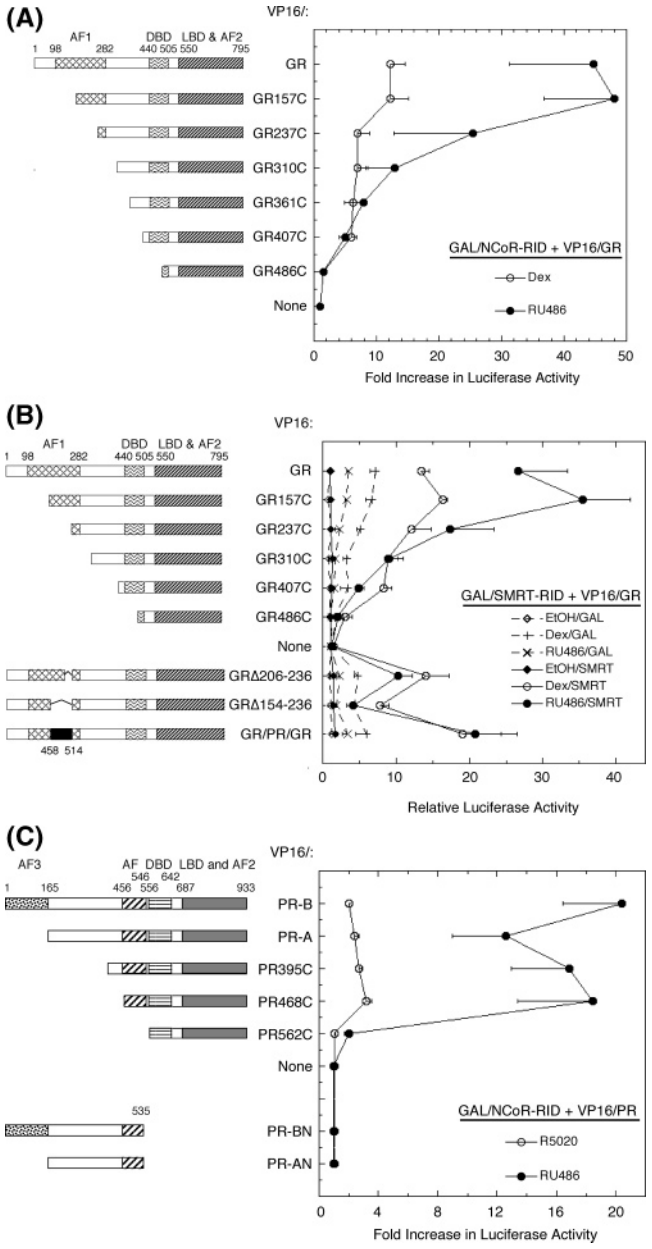

FIGURE 5: Regions of GR and PR required for the association with corepressors. Mammalian two-hybrid assay of the binding of VP16 ± various deletions of GR to either (A) GAL/NCoR-RID in Cos-7 cells or (B) GAL/SMRT-RID in CV-1 cells. Cartoons (left) show the constructs used. In B, the solid region of GR/PR/GR indicates residues 458−514 of PR-B. The assays were conducted as described in Figure 2A, after which the average values were plotted as (A) fold induction of Luciferase activity above basal activity by 1 μM Dex or RU486 (n = 3, ± SEM), or (B) normalized Luciferase activity (VP16/GR with EtOH = 1) with EtOH, 1 μM Dex, or 1 μM RU486 (n = 3−6, ± SEM). (C) Mammalian two-hybrid assay of GAL/NCoR-RID binding to VP16 ± various deletions of PR. Cartoons (left) show the constructs used. The average value from three experiments (± SEM) was plotted, as in Figure 2B, as the fold induction by 20 nM R5020 or 1 μM RU486 with the indicated VP16/PR constructs.

evokes the most robust response with the coactivator construct TIF2.0. The data for GR-NCoR contacts in CV-1 cells (not shown) were the same as those that were published (*6*) and not significantly different from those of Figure 5A in Cos-7 cells.

The data with SMRT are of additional interest because the internal deletion of amino acids 154−236 eliminates most of the interaction of GR with SMRT. Furthermore, replacing the deleted GR sequences with amino acids 458−514 in the PR-B N-terminus restores much of the function of GR with SMRT (Figure 5B). These results support our earlier demonstration that PR residues 458−514 can replace GR amino acids 154−236 to regenerate most of the interactions and biological activities of NCoR with GRs (*6*).

PR association with NCoR was studied using mammalian two-hybrid assays in Cos-7 cells (Figure 5C). Virtually all interaction activity was lost upon deletion of amino acids 468−561. However, as with TIF2.0 (Figure 4A), no binding of NCoR-RID was observed to amino terminal constructs PR-BN and PR-AN lacking the LBD but containing the above transferable module of amino acids 458−514.

Role of RIDs in NCoR Interaction with GRs and PRs. The overlap of TIF2.0 and NCoR-RID binding domains in GRs (Figure 2B vs Figure 5A) suggests that TIF2.0 competition of NCoR binding to GRs occurs via simple competitive inhibition of binding. However, the separation of the TIF2.0 and NCoR-RID binding domains in PRs (Figure 4A vs Figure 5C) raises the possibility of an alternative mechanism for PRs. This prospect is further supported by the different amino acid sequences of PR and GR regions that are required for complexation with corepressors (Figure 5B and ref *6*). If different mechanisms are involved, one might predict that different NCoR-RIDs would be involved in binding to GR versus PR. To test this hypothesis, we looked at mutants where the adjacent isoleucines are converted to alanine−alanine in RID#1 and RID#2 of NCoR-RID, a change that is known to eliminate NCoR-RID binding to nuclear receptors (*23*). We had previously shown that mutating both RIDs simultaneously eliminates corepressor binding to GRs (*14*). The data in Figure 6A and B show the consequences of mutating one RID at a time. Altering the more amino-terminal NCoR-RID#1 (NCoR-RID m1), but not the C-terminal RID#2 (NCoR-RID m2), disrupts the binding of both GRs (Figure 6A) and PRs (Figure 6B). Thus, NCoR-RID#1 is necessary and sufficient for NCoR-RID binding to both GR and PR agonist and antagonist complexes. The inactivity of the mutated NCoR-RID#1 (m1) is not due to inadequate levels of protein expression compared to that of the mutated NCoR-RID#2 (m2) (Figure 6C). Thus, despite the difference in the primary sequences of GR and PR, the same sequence of NCoR is involved in binding to both receptors.

Association of NCoR with PRE in the Presence of Progestins and Antiprogestins. To gain additional support for the above conclusion that TIF2.0 competitively inhibits corepressor interaction with PRs, we used ChIP assays to look at the binding of full-length NCoR to a PR-responsive gene in the presence of both agonists and antagonists in 1470.2 cells. We used a transiently transfected reporter driven by a progesterone response element (PRE) instead of an endogenous gene in order to be able to correlate the ChIP results with our previously documented biological responses of PR and NCoR in the same 1470.2 cells (*6*, *14*, *43*). Interestingly, sonication of the PREtkLUC reporter gave a different average fragment size than that of the cellular DNA. Sonication conditions that afforded an average size of 200−500 bps for bulk deproteinized cellular DNA by ethidium bromide staining of agarose gels yielded fragments of >580 bps but <640 bps for the transiently PREtkLUC

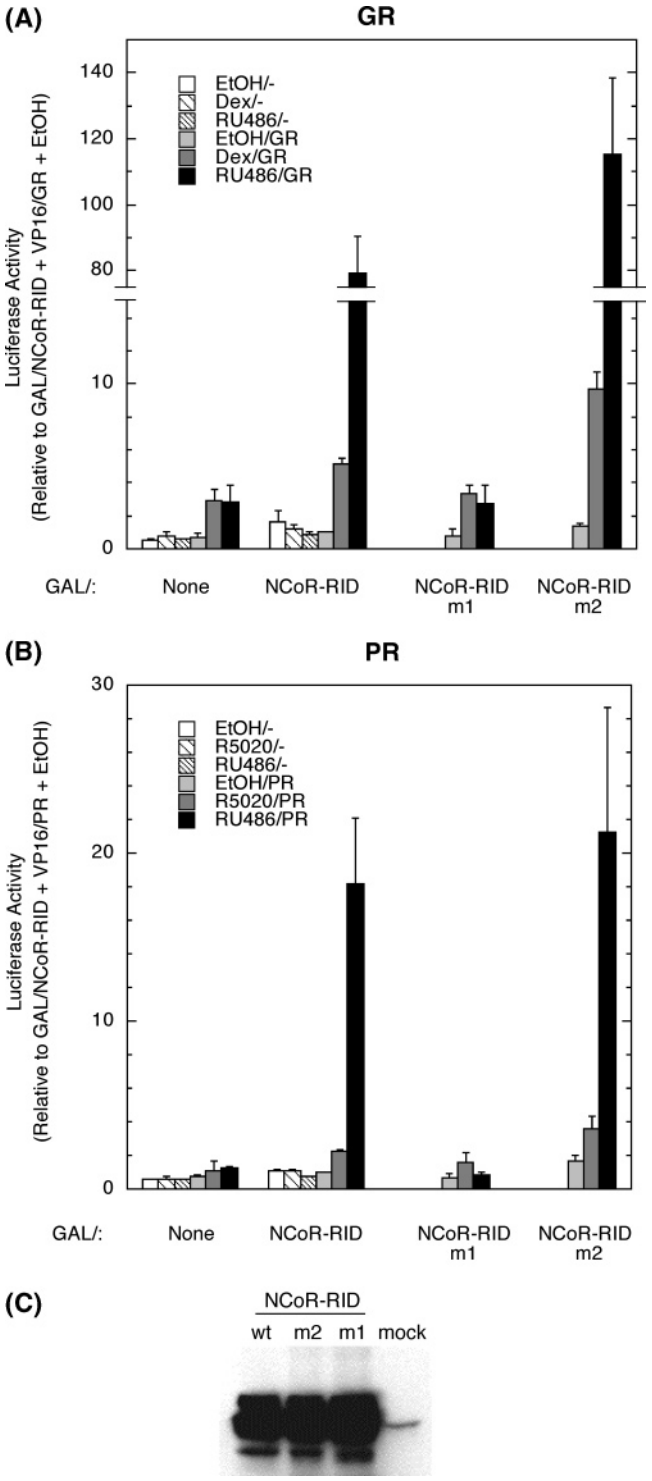

FIGURE 6: Identification of individual RID of NCoR required for the interaction with GR and PR. Mammalian two-hybrid assays were performed in CV-1 cells with the indicated GAL constructs and (A) VP16 ± GR and EtOH, 1 μM Dex, or 1 μM RU486, or (B) VP16 ± PR and EtOH, 20 nM R5020, or 1 μM RU486. After normalization (GAL/NCoR-RID with VP16/receptor and EtOH = 1), the average total Luciferase activities were plotted ($n = 3$, ± SEM). (C) Western blot of GAL/NCoR-RID#1 (m1) and -RID#2 (m2) chimeras of A and B from transiently transfected Cos-7 cells with anti-GAL-DBD antibody (Santa Cruz).

reporter, as determined by qRT-PCR with a range of differently spaced primers (data not shown). Furthermore, this average fragment size of sonicated fragments varies with the transfected plasmid and must be determined for each plasmid (Sun, Y., Wang, D., and Simons, S., unpublished results).

Four different regions of the PREtkLUC reporter, each more than 640 bps apart (Figure 7A), were examined by ChIP and quantitated by qRT-PCR for PR and NCoR binding. In addition to the PRE, to which PR is expected to bind, we included a sequence in the luciferase coding region (LucF2). Two regions upstream (4441) and downstream (Chip3) of the PRE/Luc sequences were selected to control for the nonspecific binding of proteins. Both the agonist R5020 and the antagonist RU486 promoted specific localization of PR to the PRE region but not to other segments of the PREtkLUC plasmid (Figure 7B). Similar specific localization of NCoR to the PRE was observed after treatment with both R5020 and RU486 (Figure 7C). Interestingly, though, the detection of PRE-associated NCoR in the presence of progestin is highly dependent upon the antibody used. In contrast to the anti-NCoR antibody from ABR (PA1-844) (Figure 7C), a slightly different mono-epitopic antibody from Santa Cruz (SC-1609) only weakly precipitates PRE-bound NCoR in R5020-treated cells (Figure 7D). At the same time, PRE-bound NCoR in RU486-treated cells is detected with equal efficiency by the antibodies from ABR and Santa Cruz (Figure 7D vs C). This result suggests that NCoRs associated with PR−agonist and PR−antagonist complexes have different exposed surfaces and are present in topologically different configurations.

Effect of TIF2.0 on the Association of NCoR with PRE. The above biochemical data indicate that TIF2.0 and corepressor bind to the same region of PRs complexed by both agonists and antagonists. If this competition is maintained in PR regulation of target gene expression in whole cells, we predicted that TIF2.0 would competitively inhibit NCoR association with the enhancer region of a PR-induced gene. Therefore, we asked if added TIF2.0 can reduce the amount of NCoR that is associated, in the presence of antagonist, with the PRE of the PR-regulated, transiently transfected PREtkLUC reporter in 1470.2 cells. We selected these conditions because we have already documented the biological effects of added NCoR on PR transactivation of this exogenous reporter gene in these cells (*10*, *43*). The data in Figure 8A confirm the specific localization of PR to the PRE (see Figure 7A) and establish that excess exogenous TIF2.0 has no significant effect on the amount of PRE-associated PR with the antiprogestin RU486. However, added TIF2.0 does significantly reduce the amount of NCoR that is bound to the PRE in the presence of RU486 (Figure 8B) under conditions where the amount of cellular NCoR is essentially unchanged (lane 3 vs lane 4 in Figure 8C). PCR analysis of the qRT-PCR assays shows only the expected amplified band, and no extraneous signals (data not shown), thus confirming that we are quantitating the biologically relevant species.

Effect of TIF2.0 on the Biological Effects of NCoR with PR. The data in Figure 8B lead to the prediction that added TIF2.0 should reverse the ability of NCoR to modulate the induction properties of PR in intact cells. For this reason, we used the same reaction conditions in the same 1470.2 cells as in Figure 8 to examine the effect of exogenous TIF2.0 on three induction properties of the transfected PREtkLUC reporter by added PR ± full-length NCoR (*6*, *14*, *43*): the amount of partial agonist activity of the anti-progestin Dex-

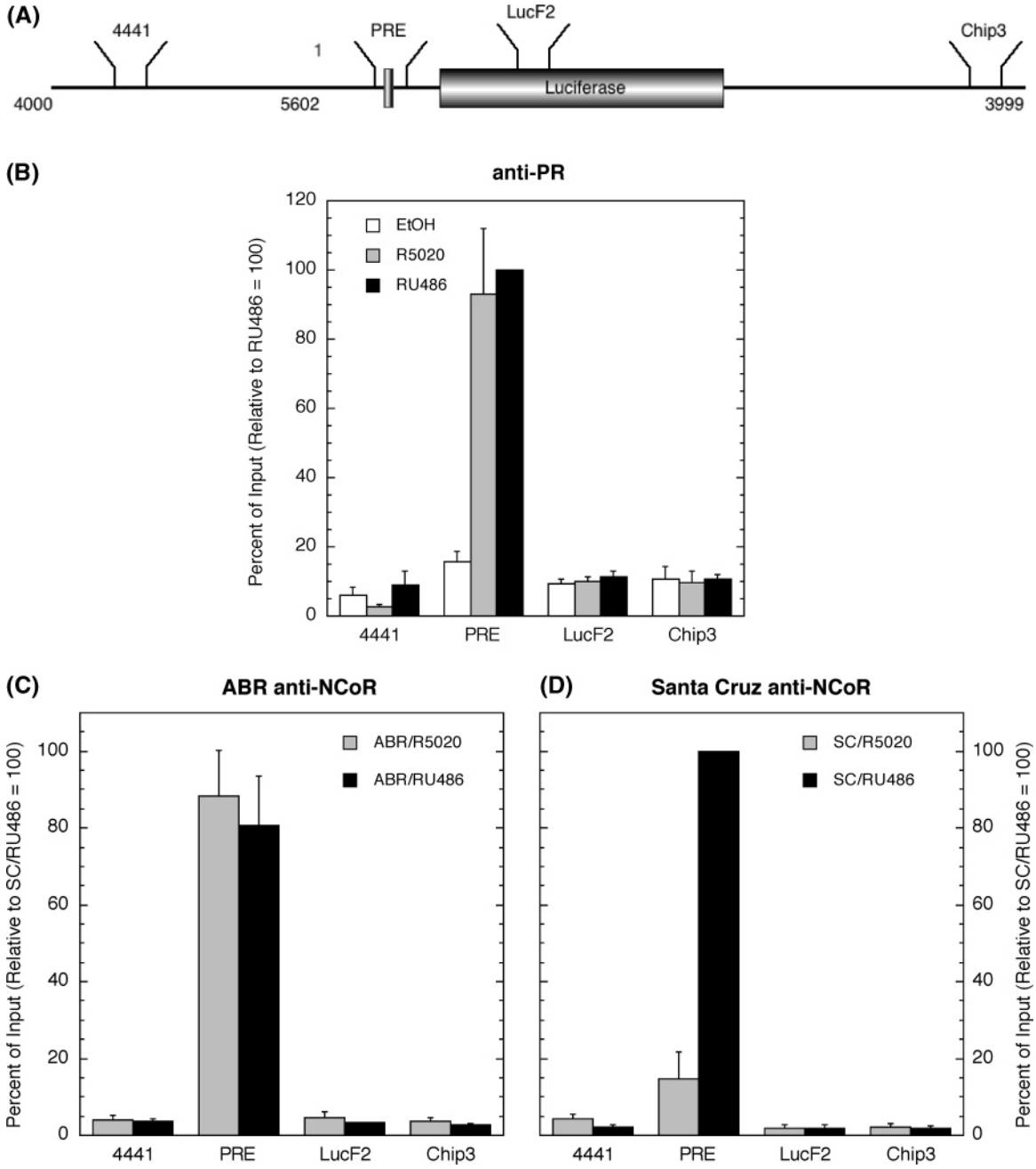

FIGURE 7: PR and NCoR binding to PREtkLUC reporter in ChIP assays. (A) Cartoon of PRE region of PREtkLUC plasmid. Location of PRE and Luciferase open reading frame are shown, with the numbers above and below the linear DNA indicating the position number in the plasmid. The Y-shaped structures abutting the DNA indicate the four regions that are amplified by PCR primer pairs. Quantitative RT-PCR was used to determine the binding of exogenous (B) PR and (C and D) NCoR to different regions of the transiently transfected PREtkLUC reporter in 1470.2 cells. Data with anti-PR antibody (B) were normalized to PR binding to PRE (as percent of input) in the presence of 1 μM RU486 = 100. Data with both anti-NCoR antibodies (C and D) were normalized to NCoR binding to PRE (as percent of input) with Santa Cruz anti-NCoR antibody (D) in presence of 1 μM RU486 = 100. In all cases, the average values of three experiments were plotted (± SEM). Binding as percent of input with RU486 ranged from 0.007 to 8%.

Mes (*10*), the fold induction of reporter gene activity above basal levels by saturating concentrations of R5020, and the position of the dose−response curve with R5020. As expected (*10*, *43*), added NCoR caused a decrease in the EC_{50} (due to a left-shift in the dose−response curve (Figure 9A and B), a rise in the fold induction (Figure 9B), and an increase in partial agonist activity (Figure 9B). When TIF2.0 is added with NCoR, the responses to NCoR alone are reversed to give values that are intermediate between those with just NCoR and just TIF2.0 (Figure 9B). Thus, TIF2.0 is capable of competing with corepressor NCoR at the levels of cell-free interactions, whole-cell localization on steroid receptor-regulated genes, and receptor-mediated biological activity.

DISCUSSION

The present study examines the molecular mechanism by which coactivators and corepressors competitively inhibit the binding of each other to steroid receptors. Our data indicate that the unexpected ability of the N-terminal region of the coactivator TIF2, TIF2.0, to compete for NCoR interactions with both GRs and PRs (*6*, *14*) is due to competition by

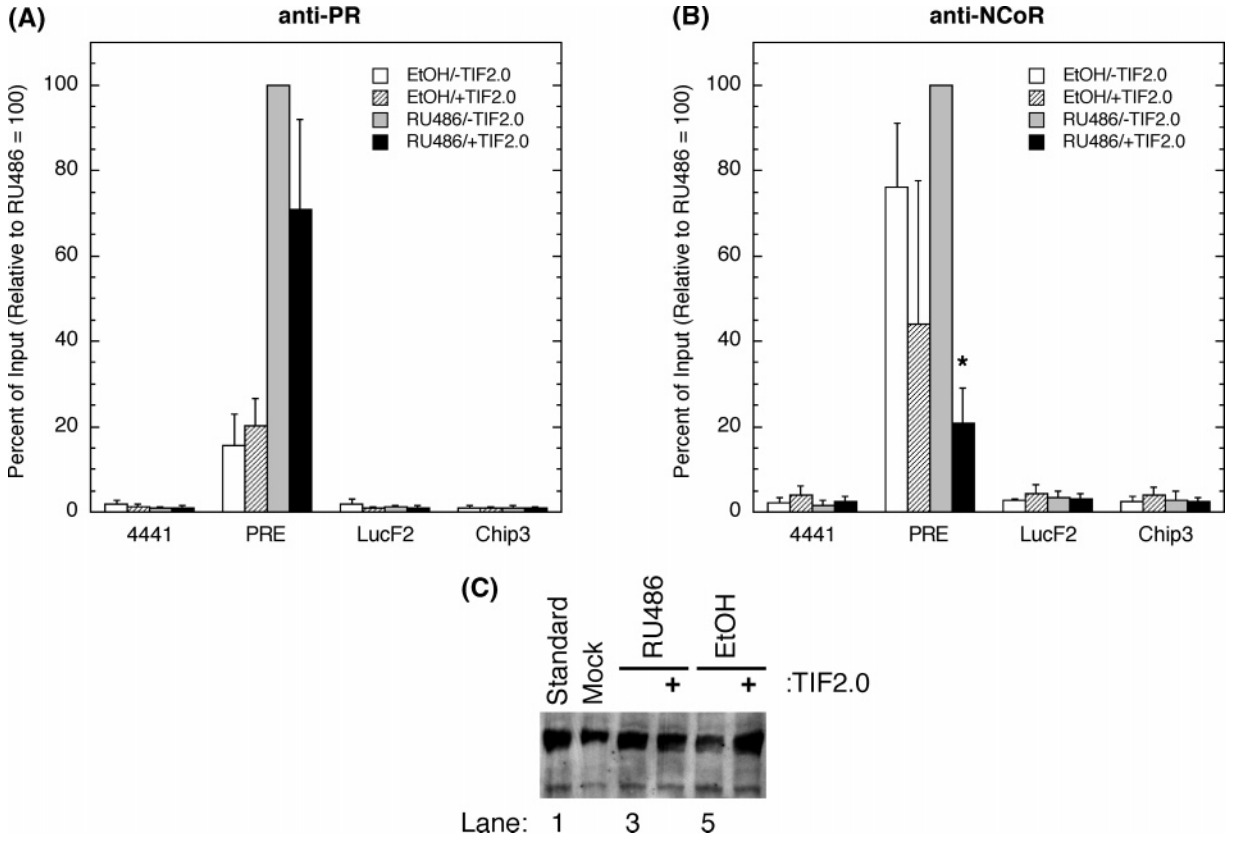

FIGURE 8: TIF2.0 inhibition of NCoR binding to PRE in ChIP assays. ChIP assays were performed and quantitated by qRT-PCR as described in Figure 7 with (A) anti-PR or (B) ABR anti-NCoR antibodies except that equimolar amounts of control plasmid (pSG5/hSA) or TIF2.0 (pSG5/TIF2.0) were added. For each antibody, the data were normalized to 100 for protein binding to PRE (as percent of input) with 1 μM RU486 in the absence of TIF2.0. The average values of three experiments were plotted ($\pm$ SEM). Binding as percent of input with RU486 ranged from 0.05 to 6%. (C) Western blot with anti-NCoR antibody (ABR PA1-844A) of equal amounts of whole cell protein from cells that had been transfected with (lanes 3−6) or without (lane 2) NCoR and with TIF2.0 (lanes 4 and 6) and then incubated with RU486 (lanes 3 and 4) or EtOH (lanes 5 and 6). Lane 1 contains a positive control for NCoR transfected 1470.2 cells.

TIF2.0 of corepressor interactions with N-terminal regions of GRs and PRs.

Pull-down, co-IP, and mammalian two-hybrid assays suggest that amino acids 157−261 of rat GR, which constitute the C-terminal half of the AF1 domain (*44*), are pivotal for the binding of TIF2.0. Because this same region of GR is required for the binding of corepressors (Figure 5B and ref *14*), competitive binding of TIF2.0 and corepressor would then result (Figure 10). N-terminal receptor sequences of PR-B are also required to bind both TIF2.0 and NCoR-RID, but the two sites in PR-B do not overlap (Figures 4A and 5C). However, the fact that the same RID of NCoR is essential for binding to non-homologous regions of PR and GR (Figure 6) suggests that the binding sites of NCoR (and TIF2.0) are determined not by primary sequence but rather by tertiary structure. Thus, it is possible for different regions of the generally unstructured N-terminal domain of PR to be in close enough proximity to mediate the binding of either TIF2.0 or corepressor but not both simultaneously (Figure 10). This hypothesis is supported by the observations that TIF2.0 competitively inhibits both the binding of NCoR to a PRE in ChIP assays (Figure 8B) and the modulatory effects of NCoR in whole-cell bioassays (Figure 9). The present results also support our earlier observations that coactivators and corepressors competitively inhibit the binding of each other to GRs under cell-free and whole-cell conditions (*14*). Finally, the current data are entirely consistent with our earlier whole-cell observations with GRs that increasing the ratio of corepressors to coactivators (and thus the amount of GR−corepressor complexes), by manipulating the ligand-bound state of thyroid hormone receptors, shifts the dose−response curve of GR−agonist complexes to the right and decreases the amount of partial agonist activity displayed by antisteroids (*14*). Therefore, biochemical and biological data fully support the model that the N-terminus of TIF2 competes with corepressors for interactions with the N-terminal sequences of GRs and PRs (Figure 10).

Accumulating evidence suggests that receptor domains in addition to the LBD are involved in binding to cofactors. However, we are not aware of any report of the N-terminal region of TIF2 binding to the GR or PR N-termini. Our results indicate that the N-terminal regions of TIF2 and GR interact (Figure 2C−E) in addition to the well-documented binding of the middle region of TIF2 (e.g., TIF2.4) with the GR LBD (*22*, *38*, *45*). The N-terminal 261 amino acids of GR are also reported to be required for the majority of VP16/NCoR-RID binding in a two-hybrid assay to GRs bound by RU486 but not Dex (*42*). This is similar to what we observe for Dex and R5020 versus RU486 in Figures 2E and 5A. The inability of TIF2.0 to bind to the N-terminus of PR-B was not anticipated but probably reflects the reduced affinity of TIF2 for PR compared to GR (*46*). The lower fold increase with added steroid for GAL/TIF2.0 binding to VP16/PR (Figure 4A) than to VP16/GR (Figure 2B), coupled with the smaller effect of exogenous TIF2 on the transactivation

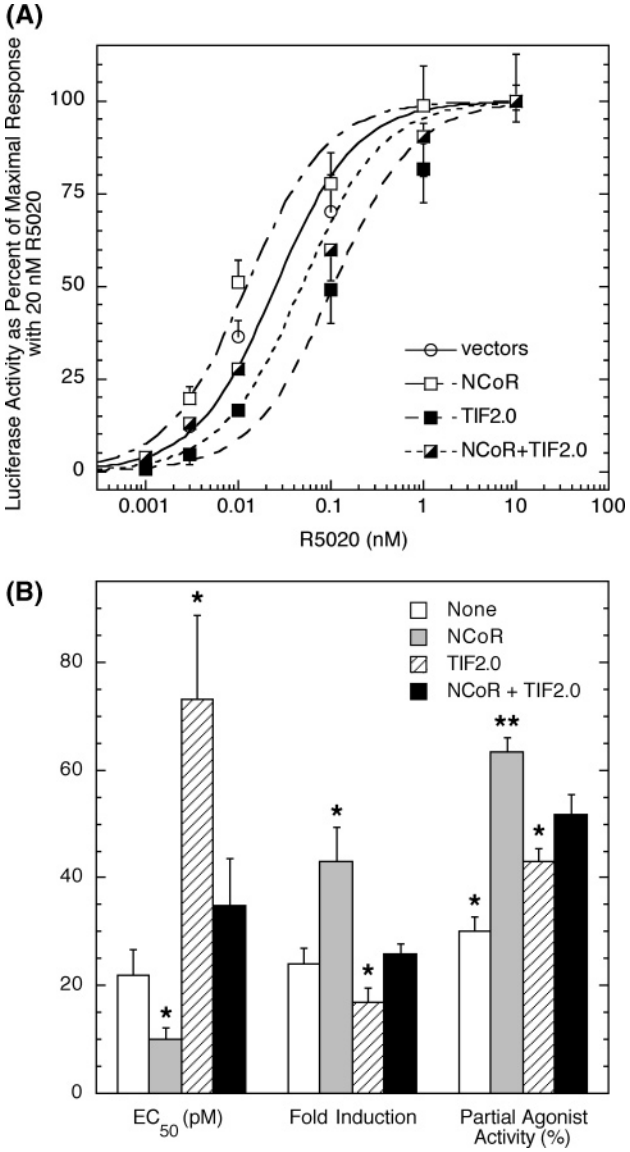

FIGURE 9: TIF2.0 competition of biological activity of NCoR with PR. (A) TIF2.0 reversal of NCoR effect on dose−response curve of PR transactivation. Triplicate samples of 1470.2 cells were transiently transfected with equimolar amounts of pCMX vector with hSA or NCoR and with equimolar amounts of pSG5 vector with hSA or TIF2.0 as indicated. After incubation with the designated concentrations of R5020, Luciferase activity (normalized to the cotransfected *Renilla* control) was expressed as percent of maximal activity with 20 nM R5020 and plotted as described in Experimental Procedures. (B) TIF2 reversal of NCoR effects on various parameters of PR-regulated gene induction. The average values from four independent experiments (± SEM) such as A for the EC_{50} for the dose−response curve with R5020, the fold induction by 20 nM R5020, and the amount of partial agonist activity of the anti-progestin Dex-Mes were determined in the presence of vector controls (None), NCoR, TIF2.0, or NCoR and TIF2.0 and then plotted as described in Experimental Procedures. The values differ significantly from those with NCoR plus TIF2.0 (filled bar) at the level of $P < 0.05$ (*) and $P < 0.001$ (**).

properties of PR (*10*) vs GR (*9*), is consistent with the hypothesis that TIF2.0 binding to the PR N-terminus is weaker than that to the GR N-terminus. Conversely, the binding of TIF2.0 to full-length PR means that C-terminal sequences of PR must contribute to this binding either directly or indirectly. The inability of PR-A to bind TIF2.0 suggests that most of the binding sequences are located in the first 165 residues of PR-B.

We consistently see negligible binding of NCoR-RID and SMRT-RID to the C-terminal regions of GR and PR in our mammalian cell two-hybrid assays (Figure 5) (*6*, *14*). A similar absence of interaction between corepressors and the androgen receptor LBD (*47*) and the requirement of both N- and C-terminal halves of AR (*48*) and ER (*49*) for NCoR binding have been reported. These results contrast with the well-documented binding of corepressors to the LBD of receptors (*20*, *50*, *51*). We suspect that these differences stem both from the contributions of N- and C-terminal receptor sequences in corepressor binding and from the lower sensitivity of the two-hybrid assay to detect the reduced affinity interactions (*52*) that would be expected to exist for corepressor binding to just one receptor domain.

The present experiments reinforce and extend the conclusions of our earlier work (*6*, *14*) that corepressor binding requires both N- and C-terminal sequences of GR and PR. This hypothesis is further consistent with the observations that both N- and C-terminal sequences are needed for the biological responses of GRs and PRs to corepressors (*43*).

Most studies of coactivator (and corepressor) binding to receptors have focused on segments of the various proteins, with the understanding that sequences relevant for the interactions of the full-length endogenous proteins may be missing. The binding of TIF2.0 to GR N-terminal domains indicates that studies of coactivator binding to GR LBD that are restricted to the LxxLL sequences of coactivator RIDs are not including all of the relevant interacting sequences. This hypothesis nicely explains why GR interactions with TIF2 versus TIF2.4 show significant differences in the interactions of RU486-bound GRs (*38*). Other examples of multiple domains mediating cofactor binding to receptors include an additional corepressor binding site in the N-terminus of ERα and ERβ (*53*), C/EBP binding to the AF1 and DBD domains of AR (*54*), and three regions of RIP140 binding to ARs (*55*). Modulator recognition factor 1 (MRF1), an AT-rich interaction domain family member, is a corepressor that binds to both ends of ERα (*56*) and cdk9, and the kinase subunit of P-TEFb binds to the N- and C-terminal regions of PPARγ (*57*). Thus, we suspect that multidomain binding of transcription factors to steroid receptors (Figure 10) may be a frequently used mechanism for generating selective and high affinity binding.

The current results offer a partial explanation for the well-known differences in gene transactivation by PR-B versus PR-A, which often inhibits PR-B actions (*58*). The N-terminal residues that are unique to PR-B have been described to neutralize the inhibitory activity of PR-A (*59*). This is the same region of PR-B that is required for most of the binding activity of TIF2.0 (Figure 4A). It has also been concluded that there are two sites for corepressor binding to full-length PRs (*37*). The N-terminal corepressor binding site identified in our study (Figure 5C) is a prime candidate for this second site. Thus, we propose that some of the transcriptional differences between PR-B and PR-A arise from the ability of TIF2 to interact with the N-terminal TIF2.0-binding site found only in PR-B and thus displace or prevent the binding of corepressors to PR-B but not PR-A (Figure 10).

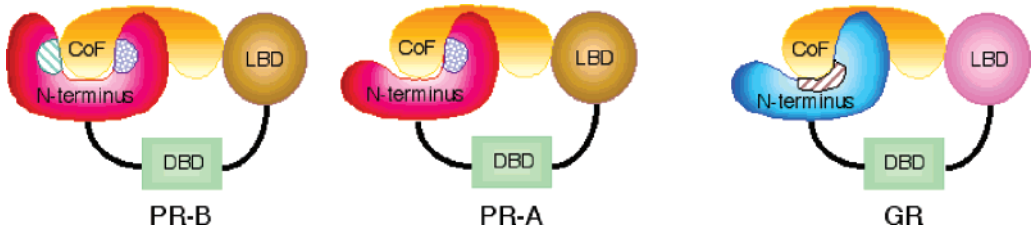

FIGURE 10: Proposed model for competitive binding of coactivators and corepressors to GRs and PRs. Both coactivator and corepressor cofactors (CoF) are envisaged as binding to sites in the LBD and N-terminus of GR and PR. The binding sites for CoFs in the GR N-terminus overlap (diagonal stripes) and are separate in the PR N-terminus (diagonal stripes and stipples) but close enough that only one CoF at a time can bind. PR-A is lacking the TIF2.0 binding site (blue diagonal stripes). See text for further details.

Our model (Figure 10) nicely complements the previously established competitive binding of coactivators and corepressors to the C-terminal LBD of receptors (*23*−*25*). Within this earlier framework, it was believed that the ability of coactivators and corepressors to have opposing, and often competitively inhibitory, effects in steroid receptor-mediated gene transcription (*6*, *9*, *12*, *14*) could be explained by the RIDs of coactivators and corepressors competing for the common binding site in the LBD of receptors. The present data indicate that both N- and C-terminal regions of coactivators and receptors are involved in this competition. Thus, we conclude that multiple regions of coactivators, corepressors, and receptors are involved in the productive biochemical interactions (Figure 10) leading to the observed biological responses of GRs and PRs.

How TIF2.0 binds to the GR and PR N-terminus is not yet clear. LxxLL motifs are commonly employed in factor binding to receptor LBDs, but there are no LxxLL motifs in TIF2.0. There are, however, several ΦxxΦΦ (where Φ = I, L, or V) and CoRNR box-like sequences in TIF2.0 that are candidates for mediating protein−protein interactions. A glutamine-rich region of SRC-1 was described as the main interaction site for binding to the N-terminal domain of ARs (*33*, *60*). However, no Q-rich region exists in TIF2.0.

Steroid is not required for the binding of TIF2.0 to N-terminal GR constructs in any assay (Figure 2D and E, and Figure 3). However, steroid is required for TIF2.0 binding to GR LBD-containing sequences in two-hybrid assays (Figure 2B) but not to full-length GR in co-immunoprecipitation assays (Figure 3A). There are two possible explanations for this. First, co-IP assays detect the binding of two proteins, whereas two-hybrid assays further require the proper conformation of the VP16 activation domain for transcriptional activation. Steroid binding to the GR LBD may influence the conformation or accessibility of the VP16 activation domain, even though it is at the other end of the GR protein, similar to how changes in DNA sequence can influence the structure of the amino terminal domain and receptor transactivation of DNA-bound receptors (*35*, *61*). GR constructs lacking the LBD would not be subject to such restrictions in the two-hybrid assay. Alternatively, the ligand-free binding of GRs and PRs to corepressors in pull-down and co-IP assays is blocked when sodium molybdate is added to prevent activation of the apo-receptors (*6*). A similar dependency of TIF2.0 binding to ligand-free receptors retaining a LBD would not be unexpected. The VP16/GR constructs lacking the LBD are constitutively activated and thus would bind independently of the whole cell activation process.

The binding of PR to the PRE in the absence of ligand (Figures 7B and 8A) was unexpected. This binding is specific as seen by the much lower binding to other reporter plasmid sequences under the same conditions. PR localization at the PRE is increased, however, upon the addition of either agonist R5020 or antagonist RU486 (Figure 7B). In contrast, NCoR association with the PRE is about the same in the presence or absence of RU486 (Figure 8B). This is similar to that observed for NCoR binding to the pS2 promoter in the absence and presence of the antiestrogen 4-hydroxy tamoxifen (*62*). The addition of estradiol decreased the localization of NCoR to the pS2 promoter, just as we observe with R5020 for NCoR on the PRE in Figure 7D. However, a different anti-NCoR antibody reveals that NCoR binding to the PRE does not change with added progestin R5020 (Figure 7C). This invariance of NCoR binding is similar to that seen for NCoR and SMRT recruitment to the enhancers of several endogenous genes by both androgens and anti-androgens (*18*). The simplest explanation for the discrepancy between the results in Figure 7C and D is that the two anti-NCoR antibodies recognize different epitopes of NCoR (or, more precisely, formaldehyde cross-linked NCoR), one of which is accessible in conjunction with both PR−agonist and PR−antagonist complexes, while the other epitope is not. This interpretation is significant because it suggests that the multi-protein assemblies with PRE-bound PR−agonist and PR−antagonist complexes have different tertiary topologies, which may contribute to the unequal transcriptional activities of the two PR complexes. These data further suggest that at least in this situation, agonist steroids may be more effective in altering the topology of the enhancer-associated corepressor than in causing the dissociation of NCoR from the enhancer region. Further experiments are required to clarify this interesting possibility.

The binding of TIF2 to NCoR (Figure 1A) confirms the finding that the related p160 coactivator AIB1/ACTR was strongly bound by NCoR and SMRT under cell-free and whole-cell conditions, whereas weaker binding was seen with SRC-1 and TIF2/GRIP1 (*41*). However, TIF2.0 does not bind NCoR, suggesting that other segments of TIF2 mediate the binding to NCoR. This is consistent with the report that the C-terminal 300 amino acids of TIF2 are involved in SMRT binding (*63*). Thus, it appears that the action of coactivators and corepressors with receptors are more complex than previously appreciated with more than one domain of TIF2 contributing to the overall activity. Not only are multiple surfaces involved in cofactors interacting with receptors such as GRs and PRs, but also the ability of TIF2 to competitively inhibit corepressor binding to receptors indicates a more

active role for coactivators and corepressors in the modification of receptor transcriptional activities than simply binding to receptors in response to the steroid present in the LBD cavity. Three-dimensional structures with full-length proteins should be very helpful in further understanding the actions of these multi-protein complexes.

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