Functional Interactions between the Estrogen Receptor and DRIP205, a Subunit of the Heteromeric DRIP Coactivator Complex*

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Nuclear receptors regulate transcription in direct response to their cognate hormonal ligands. Ligand binding leads to the dissociation of corepressors and the recruitment of coactivators. Many of these factors, acting in large complexes, have emerged as potential chromatin remodelers through intrinsic histone modifying activities. In addition, other ligand-recruited complexes appear to act more directly on the transcriptional apparatus. The DRIP complex is a 15-subunit complex required for nuclear receptor transcriptional activation in vitro. It is recruited to the receptor in response to ligand through specific interactions of one subunit, DRIP205. We present evidence that DRIP205 interacts with another member of the steroid receptor subfamily, estrogen receptor (ER). This interaction occurs in an agonist-stimulated fashion which in turn is inhibited by several ER antagonists. In vivo, a fragment of DRIP205 containing only its receptor interacting region acts to selectively inhibit ER’s ability to activate transcription in response to estradiol. These observations suggest a key role for the DRIP coactivator complex in estrogen-ER signaling.

Nuclear receptors comprise a very large family of ligand-inducible transcription factors. The ligands for nuclear receptors include steroids, retinoids, vitamin D, thyroid hormone, prostanoids, and cholesterol metabolites, such as oxysterols and bile acids. Their combined effects are vast, influencing virtually every fundamental biological process, from development and homeostasis to proliferation and differentiation. Like other eukaryotic factors that regulate transcription, nuclear receptors bind selectively to DNA, primarily as dimers through two characteristic zinc finger modules and a dimerization region that directs self-interaction or hetero-partnering. Moreover, they possess identifiable transactivation functions (AFs),1 which can independently confer activation potential to unrelated DNA-binding domains. Transactivation is mediated by both constitutive and inducible AFs (AF-1 and AF-2, respectively), the latter of which is conferred by its integral location within the ligand-binding domain (LBD).

A large number of nuclear receptor transcriptional cofactors have been isolated through interactions with receptors in yeast two-hybrid screens and from nuclear extracts in vitro, primarily using receptor LBDs as baits. One family of related proteins are collectively termed the p160 coactivators. They are represented by SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and pCIP/ACTR/AIB1 (for review, see Refs. 1–3 and references therein). Besides sequence homology, p160 proteins share an ability to stimulate ligand-dependent transactivation by nuclear receptors in transient overexpression experiments. A distinctive structural feature of the p160 coactivators is the presence of multiple LXXLL signature motifs (also called LXXD, NR boxes, or NIDs), which comprise determinants for direct interactions with the nuclear receptor AF-2 (4, 5). A second shared feature of the p160 coactivators is an intrinsic histone acetyl transferase activity and the ability to interact with other histone acetyl transferase coactivators, such as CBP/p300 and pCAF (6–8). Other cofactors can interact with some nuclear receptors in the absence of ligand and confer transcriptional repression, most notably N-CoR and SMRT through the recruitment of histone deacetylases (HDACs). These corepressors have been found as part of multiple complexes containing various HDACs through indirect interactions with corepressors bridged by mSin3a or through direct corepressor-HDAC interactions (9, 10) (11–13). Remarkably, recent data indicate that corepressors and coactivators use very similar determinants (i.e. the LXXLL motif) to recognize and interact with receptor LBDs (14–16). Ultimately, it is the absence, presence, or nature of the ligand that influences whether or not a corepressor or coactivator is bound to essentially the same site within the LBD. Functionally, these factors act in concert to modify histone tails, presumably to destabilize or stabilize chromatin (recently reviewed in Ref. 17).

In addition to actions at the level of chromatin, other, distinct nuclear receptor coactivators also appear to act directly at promoters on key components of the transcriptional apparatus. A recently discovered multisubunit complex that was found to interact with the vitamin D receptor (VDR) (18, 19) and thyroid hormone receptor (TR) (20) probably function, at least in part, at the level of direct recruitment. This complex, alternatively called DRIP or TRAP, binds to the nuclear receptor LBD AF-2 in response to ligand through a single subunit (DRIP205/TRAP220 (19, 21), also cloned as PBP (22)). However, this single subunit anchors an additional 13–15 proteins comprising the DRIP/TRAP complex, thereby conferring hormone-dependent recruitment of what appears to be a preformed complex. Other activators unrelated to steroid/nuclear receptors, such as VP16, p65 subunit of NF-κB, and SREBP-1a, also recruit this complex (called ARC; Ref. 23), and many of the
DRIP-TRAP-ARC subunits are also present in three similar, if not identical, SRB-associated complexes, NAT, SMCC, and mammalian SRB/mediator, the latter targeted by adenovirus E1A (24–26). At least seven DRIP/TRAP/ARC subunits are homologous to proteins described as components of SrB/mediator, a complex that associates with RNA polymerase II (Pol II) through its large subunit’s carboxyterminal repeat domain (reviewed in Ref. 27). This suggests that the DRIP/TRAP/ARC coactivator complex, perhaps through mediator components, functions in part by targeting polymerase II holoenzyme to promoters. In fact, these related complexes are required for transcription by these same activators, as demonstrated utilizing purified components in vitro transcription assays.

The potent effect of the DRIP complex on transcriptional activation in vitro and the generality of the activators it cooperates with suggests that it is central to the process of transcription. While preliminary data indicate that the DRIP complex interacts with several nuclear receptors, in addition to VDR and TR, such as retinoic acid receptor and peroxisome proliferator-activated receptor-γ (18, 21), its role as a ligand-dependent coactivator of steroid receptors, including estrogen receptor (ER), has not yet been clearly elucidated. We recently demonstrated that the glucocorticoid receptor (GR) LBD interacts with DRIP205 in a dexamethasone-dependent manner (28). In addition, the N-terminal transactivation function of GR, AF-1, interacts selectively with a distinct DRIP subunit, DRIP150. These results suggest that two separate activation functions might serve to bridge a multisubunit complex like DRIP through multiple interactions and perhaps offer a possible explanation for the transcriptional synergism between these two transactivation domains of GR in response to hormone.

In this work, we present evidence that DRIP205 interacts with another member of the steroid receptor subfamily, ER. This interaction occurs in an agonist-stimulated fashion, which in turn is inhibited by several ER antagonists. In transient transfection assays, a fragment of DRIP205 containing only its receptor-interacting region acts to selectively inhibit ER’s ability to activate transcription in response to estradiol. These observations suggest a key role for the DRIP coactivator complex in estrogen-ER signaling.

MATERIALS AND METHODS

Equipment and Reagents—The BIAcore 2000 system, sensor chips CM5 (certified), Tween 20, amine coupling, and GST capture kits were obtained from BIAcore Inc. For BIAcore experiments, purified recombinant human estrogen receptors α and β were obtained from PanVera Corp. The buffer used for all experiments was 50 mM Tris-Cl, pH 7.9, 150 mM NaCl, 0.2 mM EDTA, 0.05% Tween 20, 1 mM DTT. 17β-Estradiol and 4-(OH) tamoxifen were obtained from Sigma. Raloxifene was synthesized by Wyeth-Ayerst Medicinal Chemistry group. ICI-182,780 was provided by Zeneca Pharmaceuticals. Estradiol and 4-(OH) tamoxifen were obtained from Sigma. Raloxifene was synthesized by Wyeth-Ayerst Medicinal Chemistry group. ICI-182,780 was provided by Zeneca Pharmaceuticals. Drosophila melanogaster protease inhibitor (20 mM Tris, 50 mM NaCl, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM dithiothreitol, and 4 mg/ml bovine serum albumin) for 30 min at 4 °C. In vitro translated [35S]methionine-labeled (Promega TNT reticulocyte lysate system) human ER (aa 1–595), human ER-N (aa 1–251), or human ER-C (aa 185–595) were incubated with the immobilized fusion proteins for 5 min at room temperature. The beads were washed four times in the same buffer without bovine serum albumin, resuspended in 2× SDS sample buffer, and boiled for 3 min; the associated proteins were resolved by SDS-PAGE and visualized by autoradiography.

Immunoprecipitation and Immuno blotting—The U2OS-ER cell line was a kind gift from M. J. Garabedian, I. Rogatsky, and J. Trowbridge (New York University Medical Center). Cells were cultured with or without 10% fetal bovine serum (10−6 M) for 24 h. Cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM 4-(OH) tamoxifen) containing 100 μg/ml leupeptin, 1 mM benzamidine. The extracts were cleared by centrifugation for 5 min at 14,000 rpm. For ER immunoprecipitation, 20 μg of SRA1010 anti-ER IgG antibody (StressGen) was used. The samples of 10 μg of protein extract. The same amount of normal rabbit serum was used as a control. The immunoprecipitation from hormone-treated cells was performed in the presence of 10−4 M estradiol. The extracts were incubated for 2 h with the antiserum before adding a 1:1 mix of protein A and Gamma-bind G-Sepharose beads (Amersham Pharmacia Biotech) for another 1 h. The beads were washed four times with the buffer described above, washed three times with 50 mM Tris, pH 7.5, and then boiled in SDS sample buffer. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. DRIP 205 was detected by Western blot using anti-PBP polyclonal antibody against the DRIP-34 antibody (StressGen) and then stained with horseradish peroxidase-conjugated secondary antibody and chemiluminescence reagent (Amersham Pharmacia Biotech).

Protein Purification—BL21 cells expressing GST-DRIP205, GST-DRIP205 mutants, ER-DRIP derivatives, or GST-SRC1 were grown at 37 °C until A600 reached 0.4. Subsequently, the temperature was lowered to 24 °C and incubation was continued until cells reached A600 = 0.6. Protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration). Cells were allowed to grow at 24 °C for an additional 3 h, harvested by centrifugation at 5000 rpm in a Sorval SS-34 rotor for 10 min, and washed with phosphate-buffered saline buffer, containing 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitors (20 mM Tris, pH 7.5, and 1 mM dithiothreitol, 1 mM EDTA). E. coli cells were resuspended and the homogenate centrifuged for 1.5 h at 45,000 rpm in a T45 rotor (Beckman). The supernatant was applied to a glutathione-agarose column (Pierce). Bound GST-fused proteins were eluted with a gradient of reduced glutathione from 5 to 50 mM in 20 mM sodium phosphate buffer, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA. Eluted proteins were concentrated with ammonium sulfate (final concentration of 70%) and further purified by gel filtration on a Superdex-200 column.

Preparation of the Sensor Chip—Protein immobilization on a BIAcore sensor chip surface was carried out as described previously (31). Briefly, anti-GST-antibody was immobilized using an amine coupling kit, as described by the manufacturer. 6000–8000 RU of protein was typically bound. Purified GST-DRIP205 or GST-SRC1 was then immobilized using antibody-antigen interactions. Surfaces with approximately 600–800 RU of immobilized protein were used for the interaction analysis.

SPR Binding Assay and Data Analysis—Each binding cycle was performed with a constant flow of buffer at 10 μl/min. Samples of ER were injected across the surface via a sample loop. Once the injection phase was completed, the surface, the formed complex was washed with buffer for an additional 1000 s. Following each injection, the surface was regenerated with one 10-μl injection of 0.05% SDS solution. To remove immobilized GST coactivator, a 10-μl injection of 10 mM glycine, pH 2.0, was used. All experiments were performed at 25 °C. Data were collected at 1 Hz and analyzed using the BIACore evaluation program 3.0 (BIAcore, Inc.) on a Compaq PC. This program uses a global fitting analysis method for the determination of rate and affinity constants of macromolecular interactions. Refractive index differences for the ERs at different protein/ligand concentrations were adjusted using the Sigma Plot 5.0 program.

GSTM Pull-down Assays—GST-fusion proteins (20 μg), ER-LBD (aa 312–595), DRIP 205 (aa 527–774) or GST alone, immobilized on glutathione-Sepharose beads were preincubated in binding buffer (20 mM Tris, pH 7.9, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM dithiothreitol, and 4 mg/ml bovine serum albumin) for 30 min at 4 °C. In vitro translated [35S]methionine-labeled (Promega TNT reticulocyte lysate system) human ER (aa 1–595), human ER-N (aa 1–251), or human ER-C (aa 185–595) were incubated with the immobilized fusion proteins for 2 h at 4 °C. The beads were washed four times in the same buffer without bovine serum albumin, resuspended in 2× SDS sample buffer, and boiled for 3 min; the associated proteins were resolved by SDS-PAGE and visualized by autoradiography.

Immunoprecipitation and Immunoblotting—The U2OS-ER cell line was a kind gift from M. J. Garabedian, I. Rogatsky, and J. Trowbridge (New York University Medical Center). Cells were cultured with or without estradiol in the medium (10−6 M) for 24 h. Cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate buffer, 20 mM Tris, pH 7.5, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM dithiothreitol, and 4 mg/ml bovine serum albumin) for 30 min at 4 °C. In vitro translated [35S]methionine-labeled (Promega TNT reticulocyte lysate system) human ER (aa 1–595), human ER-N (aa 1–251), or human ER-C (aa 185–595) were incubated with the immobilized fusion proteins for 2 h at 4 °C. The beads were washed four times in the same buffer without bovine serum albumin, resuspended in 2× SDS sample buffer, and boiled for 3 min; the associated proteins were resolved by SDS-PAGE and visualized by autoradiography.

Estrogen Receptor-DRIP Interactions
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a ligand-dependent manner. DRIP subunits, as indicated, were used together in each pull-down assay. By expressing individual cDNAs of seven of the subunits included significantly lower amounts of detergent, we reanalyzed the ability of ER to interact with individual DRIP subunits. By expressing individual cDNAs of seven of the subunits in a coupled transcription/translation system, we observed that ERα-LBD interacted with three DRIP subunits, DRIP240, -205, and -77. However, estradiol-induced interaction occurred only with DRIP205 (Fig. 1A). This is the same subunit previously identified as interacting directly with VDR (19, 29) and TR (called TRAP220; Ref. 21) and found in a yeast two-hybrid screen as a peroxisome proliferator-activated receptor-γ-interacting murine protein called PBP (22). DRIP77 and DRIP240 also associated with ERα, albeit more weakly, and this interaction was not significantly enhanced by estradiol. The ligand-enhanced ERα interaction with DRIP205 was observed regardless of which protein was used as bait (Fig. 1B). Under the identical binding conditions described above, we found that ERβ also bound DRIP205, in a strongly estradiol-dependent manner (Fig. 1B). The DRIP205 interaction with both ER isoforms was also detected in cells using a mammalian two-hybrid assay (Fig. 1C). DRIP205 therefore does not have a binding preference for one of the two known isoforms of ER.

In order to determine if DRIP205’s interaction with ER anchors the entire multisubunit DRIP complex to the receptor, as it does with so-called class II nuclear receptors such as VDR, TR, and retinoic acid receptor, we initially attempted to view the complex bound to immobilized ERα-LBD or full-length ERα.

RESULTS

ER Interacts with the DRIP Coactivator Complex—We and others previously examined a subset of steroid/nuclear receptors for their ability to interact with the DRIP complex in the presence of their cognate ligands. In these experiments, very weak or no interaction with ER was detected (18, 21), raising the possibility that this receptor does not interact with or coactivate through the DRIP complex. However, binding conditions used in these GST pull-down assays included relatively high amounts of the detergent deoxycholate that apparently inhibited estradiol binding to ER. Using revised conditions that included significantly lower amounts of detergent, we reanalyzed the ability of ER to interact with individual DRIP subunits. By expressing individual cDNAs of seven of the subunits in a coupled transcription/translation system, we observed that ERα-LBD interacted with three DRIP subunits, DRIP240, -205, and -77. However, estradiol-induced interaction occurred only with DRIP205 (Fig. 1A). This is the same subunit previously identified as interacting directly with VDR (19, 29) and TR (called TRAP220; Ref. 21) and found in a yeast two-hybrid screen as a peroxisome proliferator-activated receptor-γ-interacting murine protein called PBP (22). DRIP77 and DRIP240 also associated with ERα, albeit more weakly, and this interaction was not significantly enhanced by estradiol. The ligand-enhanced ERα interaction with DRIP205 was observed regardless of which protein was used as bait (Fig. 1B). Under the identical binding conditions described above, we found that ERβ also bound DRIP205, in a strongly estradiol-dependent manner (Fig. 1B). The DRIP205 interaction with both ER isoforms was also detected in cells using a mammalian two-hybrid assay (Fig. 1C). DRIP205 therefore does not have a binding preference for one of the two known isoforms of ER.

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calcium phosphate method with 15 µg of total DNA including 2 µg of ERE-TK-Luc, 2 µg of CMV-β-Gal, and various amounts of pCDNA3-DRIP205 box. 12 h following transfection, cells were washed with Tris-buffered saline, and fresh medium with or without estradiol was added. Cells were harvested 24 h later, and luciferase activity was quantified using a luminometer. β-Galactosidase activity of the cell lysates was determined and used to normalize luciferase activity.

Mammalian Two-hybrid Assay—2 × 10⁴ COS-7 cells per well were seeded in Dulbecco’s modified Eagle’s medium without phenol red (Bio-Whittler) supplemented with 10% charcoal-stripped fetal calf serum. Cells were transfected with LipofectAMINE Reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. 100 ng of pGL-Luciferase reporter, 20 ng of pCMV-β-galactosidase control, 100 ng of pMGal4-DBD fused in frame to DRIP205 (527–774), and 100 ng of pVP16 fused to ERα or ERβ LBD were co-transfected per well for 4 h. Cells were incubated in appropriate hormone-supplemented Dulbecco’s modified Eagle’s medium without phenol red medium with 10% charcoal-stripped serum for 36 h and then harvested for luciferase and β-galactosidase assays. The relative level of luciferase activity was normalized to the β-galactosidase activity.

FIG. 1. A single DRIP subunit, DRIP205, binds directly to ER in a ligand-dependent manner. A, individually in vitro translated, full-length 35S-labeled DRIP subunits, as indicated, were used together with bacterially expressed and affinity-purified GST-ERα or GST in each pull-down assay. B, both ER isoforms interact with DRIP205. GST pull-down assays were performed with in vitro translated, 35S-labeled ERα or ERβ and a fragment of DRIP205 containing its nuclear receptor-interacting domains (residues 527–970) fused to GST (left panels), or in vitro translated, full-length 35S-labeled DRIP205 and full-length, bacterially expressed GST-ERα or ERβ (right panels). 10% of the input for each translated protein is shown. All pull-downs were carried out in the absence (−) and presence (+) of 1 × 10⁻⁶ M 17-β-estradiol. C, mammalian two-hybrid interaction. COS-7 cells were transfected with a GoldDBD plasmid fused to the receptor interaction region of DRIP205 and a second plasmid encoding the VP16 transactivation domain fused to ERα or β LBDs. -Fold induction in the presence of 10⁻⁶ M 17-β-estradiol was calculated with the level of relative luciferase activity in the absence of hormone set to 1. D, ERα interaction with DRIP205 in vivo is ligand-dependent. ERα was expressed stably in U2OS-ER cells and immunoprecipitated from cells untreated or treated with 1 × 10⁻⁶ M 17-β-estradiol (E2). The precipitate was separated by SDS-PAGE, blotted, and probed with an anti-DRIP205 antibody. NRS represents a parallel immunoprecipitation using a normal rabbit serum control.
bound to glutathione beads. Under these conditions, we failed to detect the DRIP complex bound to liganded ER (data not shown). However, when endogenous ERα was immunoprecipitated from human osteosarcoma U2OS cells overexpressing ERs with an anti-ER antibody and immunoblotted against antibodies directed to DRIP subunits, we observed estradiol-dependent co-precipitation of DRIP205 (Fig. 1D) in an estradiol-stimulated or -dependent fashion (Fig. 3A). As shown in Fig. 4A, the affinity of DRIP205 to ER in the presence of these antagonists was summarized in Table I.

**Table I**

| DRIP205 | ERα KD | ERβ KD |
|---------|--------|--------|
| DRIP205-F_x | 72 nM | nm |
| DRIP205-no ligand | 160 nM | 158 nM |
| DRIP205-4HT | 240 nM | 255 nM |
| DRIP205-Ral | 256 nM | 207 nM |
| DRTIP2-5-ICI | 314 nM | 329 nM |
| SRC1 | 34 nM | 25 nM |

The effects of ER antagonists on DRIP205/ER binding were also analyzed by BIAcore. Fig. 3A presents overlaid sensograms of injections of unliganded ERα and ERβ liganded with 17β-estradiol, 4-(OH) tamoxifen, raloxifene, and ICI-182,780 (all at 10^{-6} M) run over immobilized DRIP205. While binding of agonist enhanced the affinity of ERα interaction with DRIP205 compared with unliganded receptor, the pure antagonist ICI-182,780, and partial agonists 4-(OH)-tamoxifen and raloxifene all inhibited this interaction. Similar results were obtained for ERβ (data not shown). The data indicate that binding of 17β-estradiol significantly accelerated formation of the ER-DRIP205 complex, where k_{on} was enhanced more than 3-fold compared with unliganded ER and more than 10-fold relative to ER liganded with ICI-182,780. Affinity and kinetic rates of DRIP205 interactions with both ERα and ERβ in the presence of these antagonists are summarized in Fig. 3B.

Surface plasmon resonance was used to directly compare the affinities of ER with DRIP205 and SRC-1, a p160 nuclear receptor coactivator. Serial injections of ERα or ERβ liganded with 17β-estradiol were run over flow cells immobilized with equivalent amounts of bacterially expressed GST-DRIP205 or GST-SRC1. Data analysis indicated that ERα interacted with both DRIP205 and SRC1 with similar affinities, although somewhat higher for SRC1 (Table I). Essentially identical results were observed with ERβ (data not shown).

**Fig. 2. Analysis of DRIP 205/ERα binding by surface plasmon resonance.** A, overlaid injections of 30 μl of ERα at the protein concentrations of 4.4, 8.75, 17.5, 35, 70, and 140 nM over a chip surface containing immobilized GST-DRIP205 (680 RU). Prior to injection, ERα was preincubated with 1 μM (final concentration) 17β-estradiol at room temperature for 2 h. B, graph of the amount of ERα bound to DRIP205 at the end of each injection, versus logarithm of ERα concentration.
transactivation of steroid/nuclear receptors when overexpressed in transient transfection experiments. We have tested this characteristic for DRIP205 in the context of several nuclear receptors, including ER (Ref. 18 and data not shown). While we often observe modest coactivation, we believe that transient overexpression of a single subunit that derives from a multisubunit complex is not necessarily the best way to assess its functional relevance. Instead, we previously generated a small fragment of DRIP205 containing only its nuclear receptor-interacting region (Fig. 5A) (29). When cotransfected with a vitamin D-responsive reporter, this fragment, called the 205 box, selectively attenuated VDR-mediated transactivation, but not VP16 of E1A-stimulated activation (29). When tested in the osteosarcoma cell line U2OS-ER, overexpression of the 205 box resulted in a dose-dependent inhibition of ER estradiol-dependent transactivation from an ERE-regulated reporter (Fig. 5B). This dominant-negative effect was selective for ER, since it did not result in the inhibition of VP16-mediated transactivation from a GAL4-regulated reporter (Fig. 5C).

**Discussion**

Coactivators appear to play central roles in mediating steroid/nuclear receptor transactivation. Among several ligand-dependent cofactors that associate with the receptors, the DRIP complex, co-discovered as TRAP and ARC, has been shown to directly and potently coactivate ligand-dependent transcription of nuclear receptors on naked and chromatin-assembled templates in vitro. Although originally described as a TR- and VDR-interacting complex, DRIP/TRAP/ARC shares several subunits with the mammalian SRB/mediator complex and is capable of enhancing the transcriptional activation functions of several classes of activator proteins unrelated to nuclear receptors.

Despite the apparent generality of the DRIP complex, its putative role in steroid receptor signaling has not been well developed. Two subunits of the DRIP complex, DRIP205 and DRIP150, interact with the AF-2 and AF-1 domains of GR, respectively (28), suggesting that different activation surfaces specify distinct DRIP subunits. The relatively well conserved AF-2 domain among members of the steroid/nuclear receptor superfamily predicts that DRIP205 would be a common subunit interaction with the AF-2 and one that would be directly regulated by ligand binding. We have shown in this work that DRIP205 does indeed interact selectively with the ER LBD in a ligand-dependent manner that is enhanced by agonist and inhibited by antagonists such as tamoxifen and raloxifene. A fragment of DRIP205 containing only the receptor-interacting region can inhibit ER-mediated activation from a responsive reporter, indicating that this dominant negative mutant competes part or all of the DRIP coactivator complex required for estradiol-dependent transactivation by ER and utilizes the same or similar ER-interacting determinants (i.e., the LXXLL motifs) as the p160 coactivators.

Indeed, it should be pointed out that we failed to observe...
other DRIP subunits recruited to ER. In the case of several nuclear receptors, it is through the ligand and AF-2-dependent interaction with DRIP205 that the entire 15-subunit DRIP complex is recruited, and it is this complex, rather than a single DRIP subunit alone, that constitutes the functionally active DRIP species. Why we cannot detect other DRIP subunits associated with DRIP205 when the latter is recruited to ER is unclear. The biochemical conditions we utilized, although similar to what we used originally to isolate and purify the complex with VDR, may simply not be optimal in the context of ER. Alternatively, DRIP may be composed of several distinct subcomplexes, differentially recruited to steroid/nuclear receptors by DRIP205. The subfamily distinction of steroid versus nuclear receptors might also distinguish between such subcomplexes. Finally, the so-called class I steroid receptors, defined in part by their association with heat shock proteins, may require

FIG. 5. A 190-amino acid DRIP205 fragment, containing both NR boxes (205-Box) selectively inhibits ER transactivation. A, schematic diagram of 205-Box, a fragment of DRIP205 (residues 527–714) containing two nuclear receptor-interacting LXXLL motifs (NR1 and NR2). B, U2OS cells stably overexpressing ERα were transfected with a luciferase reporter plasmid containing a multimerized ERE, together with increasing amounts (in ng) of DRIP205-Box expression vector, in the absence (gray bars) or presence (black bars) of 10−6 M 17-β-estradiol (E2). Luciferase activity was normalized relative to β-galactosidase activity. C, the 205-Box has no effect on transactivation by VP16. Transient transfection assays were performed as in B except that cells were transfected with a luciferase reporter plasmid containing a multimerized Gal4-UAS enhancer, together with increasing amounts (in ng) of 205-Box and Gal4-VP16 (50 ng) expression vectors.

FIG. 4. DRIP205 associates exclusively with the ER-LBD in an AF-2-dependent manner primarily through contributions of NR box 1. A, schematic representation of full-length ER, ER-N (amino acids 1–251), and ER-C (amino acids 185–595). B, DRIP205 interacts with the ER-LBD only. ER, ER-N, or ER-C were in vitro transcribed and translated and incubated with Sepharose beads containing bound GST-DRIP205, in the presence or absence of 17-β-estradiol. GST beads were used as a control for nonspecific binding. Bound ER, ER-N, or ER-C was resolved by SDS-PAGE and visualized by autoradiography. C, ER/DRIP205 interaction is AF-2-dependent. GST pull-downs of intact ERα or ERα containing AF-2 Leu to Ala mutations at residues 539 and 540 were carried out as described for B. D, relative contributions of NR boxes. GST pull-down assays using in vitro translated ERα (lanes 1–11) or VDR (lanes 12–22) in the absence or presence of 10−6 M 17-β-estradiol or 1,25-dihydroxyvitamin D3, respectively, together with 2 μg of GST-DRIP205-(527–970) (wt; lanes 2 and 3 and lanes 13 and 14), GST-DRIP205ΔNR1 (residues 604–774; lanes 4 and 5 and lanes 15 and 16), or GST-DRIP205ΔNR2 (527–604; lanes 6 and 7 and lanes 17 and 18) are shown. Mutants 1 and 2 (lanes 8–11) correspond to GST-DRIP205-(527–970) containing point mutations in either NR1 or NR2 boxes, respectively, that change each LXXLL motif to LXXAA. GST proteins used in the experiments depicted in D were quantitated by visualization on Coomassie-stained SDS-PAGE.
additional factors, perhaps cytosolic, for proper DRIP complex association that are missing from our nuclear extract preparations.

We have previously reported that the same ER ligands examined here have similar potentiating or inhibiting effects on the binding of SRC3 (ACTR/AIB-1) to ERs (36). Moreover, the DRIP and p160 classes of coactivators share very similar determinants for binding to steroid and nuclear receptors (i.e. the AF-2 domain in the receptor and the LXrXLL motifs in the coactivator). This raises the question of how the binding of these coactivators is regulated vis-a-vis each other, if, in fact, they are at all. We and others have previously proposed a sequential model whereby the CBP/p160 complex modification of histones leads to chromatin remodeling, which in turn renders the preinitiation complex accessible to recruitment by the DRIP complex (17, 21). A second possible scenario is a combinatorial type of mechanism, whereby both complexes are recruited separately by two bound receptor heterodimers on the same promoter, functioning in concert to activate. Very recent results of Chen and co-workers (37) demonstrated that acetylation of SRC3/ACTR by p300/CBP actually disrupts the former’s interaction with ER; this could conceivably allow the DRIP complex to bind ER as a second step in the activation process.

ER ligands that in some cell types exhibit agonist activity, such as 4-OH-tamoxifen and raloxifene, inhibit ER/DRIP205 interaction in vitro. Similar results have also been observed for ERαs and ERβ interactions with SRC1, SRC3, GRIP1, and CBP.3 The fact that a compound that consistently inhibits ER-coactivator interactions can in some circumstances stimulate ER-mediated transactivation suggests that unidentified ER-interacting factors might mediate the activity of these compounds in modulating ER function, perhaps through additional activation domains in the ER (i.e. AF-1). It will be interesting to determine whether or not additional DRIP subunits interact with ER-AF-1, as is the case with DRIP150 and GR (28), and whether or not in the context of full-length ER partial agonists have differential effects on this interaction in vivo.

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