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Different Classes of Coactivators Recognize Distinct but Overlapping Binding Sites on the Estrogen Receptor Ligand Binding Domain*

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We have analyzed interaction of coactivators with the wild-type estrogen receptor α (ER), HEG0, and a mutant, L536P-HEG0, which is constitutively active in several transiently transfected cells and a HeLa line that stably propagates an estrogen-sensitive reporter gene. Different classes of coactivators do not recognize the ER ligand binding domain (LBD) in the same manner. Steroid receptor coactivator-1 (SRC-1), amplified in breast cancer-1 (AIB-1), transcriptional intermediary factor-1 (TIF-1), transcriptional intermediary factor-2 (TIF-2), and receptor interacting protein 140 (RIP140) interacted with HEG0 and L536P-HEG0 in the presence of estradiol, but generally not in the presence of anti-estrogens. However, ICI164,384 stimulated some interaction of RIP140 with LBDs. SRC-1, AIB-1, and RIP140 interacted constitutively with the L536P ER, whereas TIF-1 and TIF-2 interacted only weakly in the absence of hormone. Reciprocal competition for binding to the ER LBD was observed between different classes of coactivators. Moreover, coexpression of RIP140 blocked enhanced transactivation by HEG0 observed in the presence of TIF-2, suggesting that RIP140 may play a negative role in ER signaling. We conclude that constitutive activity of L536P-HEG0 is manifested to similar degrees in different cell types and likely arises from constitutive coactivator binding; different classes of coactivators recognize distinct but overlapping binding sites on the ER LBD. Finally, the observation that L536P-HEG0 interacted constitutively with AIB-1, a coactivator that has been implicated in ER signaling in breast and ovarian cancer, suggests that similar mutations in the ER may contribute to hormone-independent proliferation of breast and ovarian cells.

The estrogen receptor (ER)¹ is a member of the family of nuclear receptors (1–6). Similar to other nuclear receptors, the ER is a ligand-activated regulator of transcription that functions through stimulating formation of transcriptional preinitiation complexes. Preinitiation complexes include general transcription factors, RNA polymerase II, and multiple components of polymerase II holoenzyme (7–10). The ER stimulates the assembly of these components through interaction with factors collectively known as coactivators that interact with the receptor ligand binding domain (LBD) in the presence of hormone (11). This interaction requires the AF-2 activating function, located at the extreme C terminus of the LBD.

Different classes of coactivators have been identified, including SRC-1 and related proteins such as pCIP/AIB-1/RAC3 (12–16), TIF-2/GRIP-1 (17, 18), RIP140 (19), TIF-1 α and - β (20, 21), all of which apparently interact with receptors through signature motifs containing the core LXXLL (14, 21, 22). The cointegrator CREB binding protein (CBP) also binds directly to receptor ligand binding domains as part of a ternary complex with members of the SRC-1 family (13, 14, 23). CBP and SRC-1 have been shown to stimulate acetylation of histones, consistent with observations that transcriptional activators stimulate remodeling of chromatin, thus facilitating access of general transcription factors to initiation sites (24, 25).

Evidence is accumulating that suggests that different coactivators do not function in the same manner. CBP interacts with SRC-1 and related proteins, but not with coactivators of 140 kDa (13). SRC-1, AIB-1, and TIF-2 stimulate hormone-dependent transactivation by nuclear receptors in transient transfection assays (12, 15, 18), whereas RIP140 and TIF-1 function weakly or not at all under these conditions (19, 21). However, active templates in transient transfection assays may not require extensive chromatin remodeling and, thus, all of the components essential for preinitiation complex formation on endogenous genes. Indeed, a number of proteins likely to be required for chromatin remodeling have been shown to interact with members of the TIF-1 family (21).

Given the evidence that different coactivators may function by distinct mechanisms, it is possible that recruitment of several coactivators to a target gene would stimulate preinitiation complex formation through multiple pathways. We were inter-

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¹ The abbreviations used are: ER, estrogen receptor α ; AIB-1, amplified in breast cancer-1; ERE, estrogen response element; GRE, glucocorticoid response element; GST, glutathione *S*-transferase; LBD, ligand binding domain; RIP140, receptor interacting protein 140; SRC-1, steroid receptor coactivator-1; TIF-1, transcriptional intermediary factor-1; TIF-2, transcriptional intermediary factor-2; EBV, Epstein-Barr virus; CBP, CREB binding protein; CAT, chloramphenicol acetyltransferase; OHT, hydroxytamoxifen.

ested in analyzing the interaction of coactivators singly and in combination with the LBD of the ER. Using ligand binding domains from the wild-type receptor and a constitutively active ER mutant L536P (26), we found that, whereas different coactivators do not recognize the LBD in the same way, they do compete for interaction with the ER, indicating that they recognize distinct but overlapping binding sites.

MATERIALS AND METHODS

Plasmid Construction—The estrogen-sensitive episomal reporter plasmid ERE3-TATA-CAT/EBV is identical to the previously described GRE5-CAT/EBV (27), except that the five glucocorticoid response elements (GREs) of GRE5-CAT/EBV were replaced by three consensus estrogen response elements (EREs) from the *Xenopus* vitellogenin gene. As described previously (27), vectors of this type carry a gene conferring resistance to hygromycin and replicate stably in HeLa cells in the presence of hygromycin. Plasmids encoding full-length SRC-1, mouse TIF-1 (residues 1–750), TIF-2 (residues 624–1287), and RIP-140 (residues 733–1158) were generous gifts of Pr. P. Chambon (Illkirch, France). A cDNA clone encoding AIB-1 was isolated from a yeast two-hybrid screen of a human fetal kidney library. The fragment of AIB-1 (residues 518–813) used in GST pull-down experiments contains three LXXLL signature motifs. Coactivator expression vectors were constructed by inserting cDNAs encoding full-length RIP140, full-length TIF-2 or AIB-1 (residues 513–813) in the expression vector pSG5.

Cell Culture and Transfections—All cells were grown in 10 cm plates. Ishikawa and MCF-7 cells were propagated in α minimal essential medium plus 5 and 10% fetal bovine serum, respectively. COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. HeLa cells propagating the ERE3-TATA-CAT/EBV vector (HeLa/ERE3-EBV) were generated essentially as described (27). Briefly, cells were transfected at 50% confluence with 15 μ g of ERE3-TATA-CAT/EBV using the calcium phosphate coprecipitation technique. Medium was changed after 24 h, and 24 h later hygromycin was added to a final concentration of 250 μ g/ml. Medium was changed every 48 h, and when all cells on control untransfected plates had been killed off, hygromycin-resistant cells were split and expanded in the presence of 100 μ g/ml hygromycin. Transient transfections were performed by lipofection (COS-7) with Lipofectin (Life Technologies, Inc.) with 50 ng of either HEG0/pSG5 or L536P-HEG0/pSG5 expression vectors, which encode the wild-type and mutant receptors, respectively, 250 ng of the reporter plasmid pXP-ERE3, and 500 ng of the internal control *lacZ* expression vector p610AZ. Alternatively, transfections were performed by calcium phosphate coprecipitation (Ishikawa, MCF-7, and HeLa) with 1 μ g of either HEG0/pSG5 or L536P-HEG0/pSG5, 2 μ g of ERE3-TATA-CAT/EBV reporter plasmid, and 2 μ g of RSV-*lacZ* as an internal control for transfection efficiency and 15 μ g of Bluescript (Stratagene) carrier DNA. HeLa/ERE-EBV cells were transiently transfected by calcium phosphate coprecipitation with 1 μ g of either HEG0/pSG5 or L536P-HEG0/pSG5, 2 μ g of RSV-*lacZ* internal control, pSG5-based coactivator expression vectors added as indicated in the figures, and Bluescript carrier DNA to 20 μ g. Prior to transfections, cells were grown in medium containing charcoal-stripped serum for 24 h. Medium was changed 24 h after transfection, and ligand was added (estradiol, 10 nM; hydroxytamoxifen and ICI164384, 100 nM) for 24 h. Cells were harvested, and luciferase or CAT activity was determined. CAT activity was determined using the solvent extraction method. Assays for β -galactosidase were performed as described (6).

Western Blotting—Western analysis of ER expression levels in extracts of transiently transfected HeLa/ERE-EBV cells were performed as described (26) using the B10 anti-ER monoclonal antibody.

Production of GST-ER Fusion Proteins—GST-ER expression vectors were constructed by inserting fragments encoding the wild-type ER LBD (residues 270–595) or that of L536P-HEG0 (26) into the *Eco*RI sites of pGEX2TK (Amersham Pharmacia Biotech). GST-LBD fusion proteins were expressed and purified from *Escherichia coli*. Briefly, bacteria expressing the GST fusion proteins were grown at 37 °C to an A_{595} of 0.5. GST-ER expression was induced for 3 h at 30 °C with 0.5 M isopropyl β -D-thiogalactopyranoside. Cells were centrifuged, and the bacterial pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 μ M ZnCl₂, 0.1 mM dithiothreitol, 10% glycerol, 100 mg/ml lysozyme, and protease inhibitors) and lysed by sonication four times. Aliquots of cleared bacterial lysates were loaded on SDS-polyacrylamide gels, and expression levels of fusion proteins were determined by Western analysis with anti-ER monoclonal antibody B220.

Coupled *In Vitro* Transcription and Translation—Transcripts encoding SRC-1, AIB-1, TIF1, TIF2, and RIP140 were transcribed from the

corresponding expression vectors with T7 or T3 RNA polymerase and translated *in vitro* with a TNT coupled reticulocyte lysate system (Promega) in the presence of [³⁵S]methionine according to the manufacturer's instructions. Translation efficiency was determined by running 0.5 μ l of the reaction on an SDS-polyacrylamide gel.

GST Pull-down Assays—GST-ER fusion proteins were purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) by incubating 1 ml of crude bacterial extract with 200 μ l of beads overnight at 4 °C. After three washes with GST buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.3 mM dithiothreitol, 5% glycerol, 0.1% Nonidet P-40, and protease inhibitors) the amount of bound protein was determined by the Bradford method. Beads containing GST-ER fusion protein or GST alone were incubated with *in vitro* translation products in the presence of vehicle or hormone at 4 °C for 2 h. The beads were then washed three times with 800 μ l of GST buffer, boiled for 2 min in 2 \times SDS-polyacrylamide gel sample buffer and electrophoresed on an SDS-10% polyacrylamide gel. Gels were then fixed with 30% (v/v) methanol, 10% (v/v) acetic acid for 1 h, treated with EN³HANCE (DuPont) for 20 min, and vacuum-dried. Autoradiography was performed overnight.

RESULTS

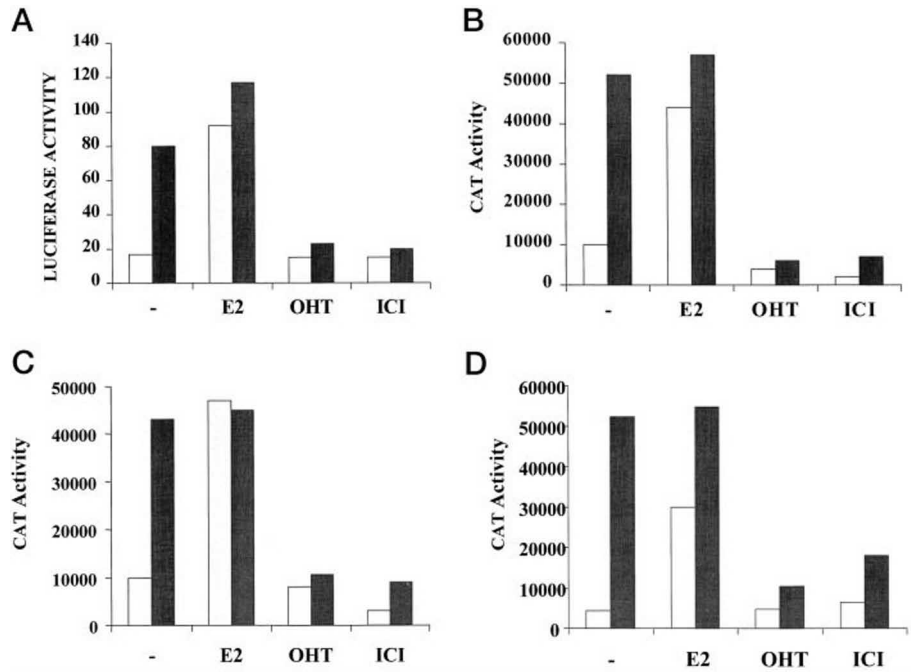
Constitutive Activity of L536P-HEG0 in Transiently and Stably Transfected Cells—We have previously identified an ER carrying a point mutation in the LBD, L536P, located immediately N-terminal to the AF-2 activating domain (26). This mutation causes a conformational change in the LBD that partially mimics that induced by hormone and renders the receptor constitutively active when transiently expressed in COS-7 cells (Fig. 1A). We were interested in determining whether L536P-HEG0 displayed comparable levels of constitutive activity in different cell types, particularly those expressing endogenous ER. The human endometrial and breast carcinoma cell lines Ishikawa and MCF-7, respectively, were transiently transfected with an estrogen-sensitive CAT reporter plasmid, ERE3-TATA-CAT, and an expression vector for either HEG0 or L536P-HEG0. In both Ishikawa and MCF-7 cells, expression of L536P-HEG0 gave rise to high levels of hormone-independent transactivation when compared with the wild-type receptor (Fig. 1, B and C). In both cases, transactivation in cells expressing ER expression vector was severalfold higher than that stimulated by the endogenous ER (data not shown). As expected, ER antagonists hydroxytamoxifen (OHT) and ICI164,384 did not stimulate transcription by HEG0 in either Ishikawa or MCF-7 cells (Fig. 1, B and C), and both OHT and ICI164,384 repressed the constitutive activity of L536P-HEG0 in these cells. Similar results to those presented in Fig. 1, B and C, were obtained in transiently transfected HeLa cells (data not shown).

Given the evidence that coregulators can stimulate chromatin remodeling during preinitiation complex formation, it was of interest to determine whether L536P-HEG0 displayed similar levels of constitutive activity on a DNA template in stably transfected cells. Therefore, a stably transfected HeLa cell line was created by introduction of an ERE3-TATA-CAT cassette inserted in an EBV episomal vector to create (HeLa/ERE-EBV). Similar EBV vectors have been used successfully to stably propagate cloned cDNA sequences in HeLa cells (27, 28). Since HeLa cells do not express significant levels of ER, the function of L536P-HEG0 could be monitored in HeLa/ERE3-EBV cells transiently transfected with a receptor expression vector. Under these conditions (Fig. 1D), L536P-HEG0 displayed high levels of constitutive activity, similar to that observed in transiently transfected COS-7, Ishikawa, MCF-7, and HeLa cells (Fig. 1, A–C, and data not shown). Thus, the constitutive activity of L536P-HEG0 is manifested in a variety of cell types on both transiently transfected and stably propagated DNA templates.

Coactivators Interact Differently with the Ligand Binding Domain of L536P-HEG0—The constitutive function of the

FIG. 1. Constitutive transactivation by the ER LBD mutant L536P-HEG0.

A–C, transient transfections of ER expression vectors HEG0 (stippled bars) or L536P-HEG0 (black bars) along with estrogen-sensitive reporter plasmids ERE3-TATA-luciferase (A) or ERE-TATA-CAT (B and C) in COS-7 (A), Ishikawa (B), and MCF-7 cells (C). Transfected cells were treated with ethanol vehicle (–), 10 nM estradiol (E2), 100 nM hydroxytamoxifen (OHT), or 100 nM ICI164,384 (ICI) as indicated. D, transient transfection of a HeLa cell line stably propagating an estrogen-sensitive Epstein-Barr virus episomal vector ERE3-TATA-EBV with expression vectors for HEG0 (light bars) or L536P-HEG0 (dark bars) as indicated. Results of three sets of experiments are presented. Values varied by $\pm 20\%$.



L536P-HEG0 LBD was probed further by performing GST pull-down assays with GST fusion proteins composed of either the wild-type or L536P ligand binding domains and coactivators *in vitro* translated in the presence of [35 S]methionine. All coactivators, SRC-1, AIB-1_{518–813}, RIP140_{733–1158}, and TIF-2_{624–1287}, bound to the LBD of HEG0 in a hormone-dependent manner (Fig. 2, A–D). However, significant binding of TIF-1 to HEG0 was only seen with large amounts of *in vitro* translated protein (Fig. 2E, lane 4). OHT did not stimulate coactivator binding to the HEG0 LBD (Fig. 2, A–E, lane 5). Significantly, however, whereas ICI164,384 had no effect on binding of SRC-1, AIB-1_{518–813}, TIF-1, and TIF-2_{624–1287} to the receptor (Fig. 2, B–E, lane 6), it did stimulate some binding of RIP140 to the HEG0 LBD (Fig. 2A, lane 6).

The interaction of coactivators with the L536P-HEG0 LBD in the absence of ligand varied widely. SRC-1, AIB-1_{518–813}, and RIP140_{733–1158} bound the mutant LBD to similar degrees in the presence and absence of estradiol, whereas only low levels of binding of TIF-1 and TIF-2_{624–1287} were observed in the absence of ligand (Fig. 2, A–E, lanes 7 and 8). Full-length AIB-1 also interacted constitutively with the L536P receptor (data not shown). It is noteworthy that TIF-2 and SRC-1 have similar domain structures and yet do not recognize the L536P-HEG0 LBD in the same way. In the presence of estradiol, TIF-1 bound L536P-HEG0 at significantly higher levels than to the wild-type receptor (Fig. 2E, lane 8). Consistent with gene transfer experiments, OHT repressed coactivator binding to L536P-HEG0 (Fig. 2, A–E, lane 9). ICI164,384 repressed binding of SRC-1, AIB-1_{518–813}, and TIF-2_{624–1287} and did not stimulate binding of TIF-1 to the L536P-HEG0 LBD (Fig. 2, B–E, lanes 10). However, similar to HEG0, the L536P-HEG0 LBD bound significant levels of RIP140_{733–1158} in the presence of ICI164,384 (Fig. 2A, lane 10). Taken together, these results indicate that different coactivators do not recognize the ligand binding domain of the ER in the same manner.

The capacity of RIP140, SRC-1, AIB-1 and TIF-2 to modulate basal and hormone-stimulated transactivation by the wild-type ER and L536P-HEG0 on a stably propagated episomal template was analyzed by transient transfection of receptor expression vectors with expression vectors encoding full-length coactivator cDNAs in HeLa/ERE3-EBV cells. Cotransfection of

increasing amounts of a RIP140 expression vector had no or only a minor stimulatory effect on expression of luciferase activity in the presence of either HEG0 or L536P-HEG0, but inhibited both basal and hormone-stimulated transactivation at high concentrations (Fig. 3, A and B). These results suggest that potential chromatin remodeling activity of RIP140 is not revealed by transfection of cells carrying stably propagated reporter genes. SRC-1 stimulated basal and hormone-stimulated activation by both wild-type and mutant receptors 2–3-fold (Fig. 3, C and D). Similar, although somewhat more modest, effects were observed with full-length AIB-1 (Fig. 3, E and F). Cotransfection of TIF-2 strongly stimulated both hormone-independent and hormone-dependent transactivation by the wild-type ER at the highest levels of expression vector tested (Fig. 3G). TIF-2 had a somewhat more modest effect on hormone-dependent activation by L536P-HEG0, although at lower concentrations, and stimulated activation only weakly in the absence of hormone (Fig. 3, G and H).

Control Western analyses of transiently transfected cell extracts used in Fig. 3 showed that ER levels did not vary significantly when coexpressed with coactivators. Thus, altered levels of transactivation by the wild-type and L536P ERs observed in the presence of coexpressed coactivators were not due to effects of coactivators on expression of the ER (Fig. 4). Taken together, the results of Figs. 2 and 3 suggest that the activity of L536P-HEG0 in the absence of hormone is manifested, at least in part, by its constitutive interaction with the widely expressed coactivator AIB-1 and/or other members of the SRC-1 family.

Competition of Different Coactivators for Binding to the ER LBD—Given the differential interaction of heterologous coactivators with the ER ligand binding domain, it was of interest to determine whether multiple coactivators competed with each other for binding to the receptor. Competition assays were set up where interaction of coactivators SRC-1, AIB-1, TIF-2, and RIP140 was analyzed in the presence of limiting quantities of GST-HEG0 LBD. This was determined by serial dilution of GST-HEG0 LBD beads with GST beads. Thus, serial dilution of 10 μ l of GST-HEG0 LBD beads (2.5 μ g of protein) with GST beads led to reduced binding of *in vitro* translated SRC-1, AIB-1, RIP140, or TIF-2 in the presence of estradiol (data not

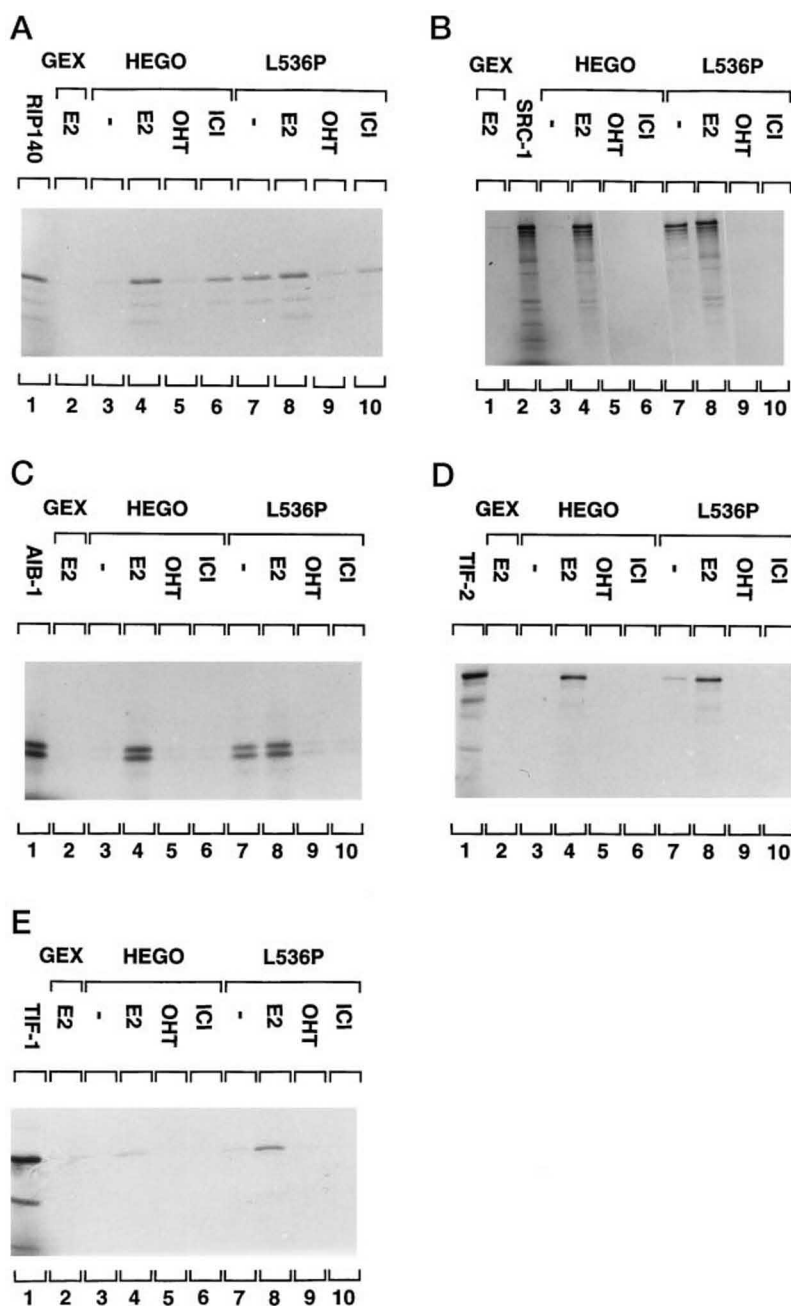


FIG. 2. GST pull-down experiments with GST-ER LBD fusion proteins and *in vitro* translated coactivators. A–D, experiments were performed with free GST control (*GEX*) or GST fusion proteins containing the LBD from the wild-type ER (*HEG0*) or the mutant L536P-*HEG0* (*L536P*) and *in vitro* translated RIP140_{733–1158} (A), SRC-1 (B), AIB-1_{518–813} (C), TIF-2_{624–1287} (D), and TIF-1 (E) in the absence of hormone (–), in the presence of 10 nM estradiol (E2), 100 nM hydroxytamoxifen (OHT), or 100 nM ICI164,384 (ICI) as indicated. An aliquot (0.5 μ l) of *in vitro* translation product is shown in lane 1 (lane 2 in B).

shown). Based on these experiments, 64-fold dilutions of GST-*HEG0* (0.039 μ g of GST-*HEG0*) were used in reciprocal competition experiments for binding of RIP140, AIB-1, TIF-2, and SRC-1 to the *HEG0* LBD.

Competition assays were performed using 0.5 μ l of *in vitro* translated coactivator, and increasing volumes (1 to 20 μ l) of competitor *in vitro* translated coactivator, such that competitor was present in up to 40-fold excess. Under these conditions, RIP140_{733–1158} and SRC-1 competed with each other for binding to the ER LBD (Fig. 5, A and B). In a separate control experiment, 20 μ l of control *in vitro* translation extract expressing LacZ had no effect on binding of RIP140_{733–1158} or SRC-1 to the ER LBD (Fig. 5, A and B, lanes 11 and 12), thus indicating that the observed competition between RIP140_{733–1158} and SRC-1 was specific. Reciprocal competition was also observed between RIP140_{733–1158} and TIF-2_{624–1287} (Fig. 5, C and D). In another set of experiments, increasing amounts of AIB-1_{518–813} competed for binding of RIP140_{733–1158} (Fig. 6A), SRC-1 (Fig. 6C), and TIF-2_{624–1287} (Fig. 6E) to the ER LBD.

Whereas RIP140_{733–1158} competed for binding of AIB-1_{518–813} (Fig. 6B), SRC-1 (Fig. 6D) or TIF-2_{624–1287} (Fig. 6F) competed only weakly. Reciprocal competition between coactivators suggests that they have similar affinities for the ER LBD in these experiments. We also found that, whereas heterologous coactivators disrupted interaction of TIF-1 with the ER LBD, TIF-1 did not compete for their binding (data not shown). This is consistent with the observation that high levels of TIF-1 were required to observe significant interaction with the *HEG0* LBD (Fig. 2). In summary, whereas our data indicate that different classes of coactivators do not bind to the receptor in the same manner, the results of competition assays suggest that SRC-1, AIB-1, RIP140, TIF-1, and TIF-2 recognize overlapping binding sites on the ER LBD.

The results of Figs. 5 and 6 suggest that coactivators may compete for binding to the ER *in vivo*. We examined the potential of RIP140 to compete for stimulation of ER-dependent transactivation by TIF-2 in HeLa-ERE3/EBV cells. Consistent with results described above (Fig. 3), TIF-2 expressed from

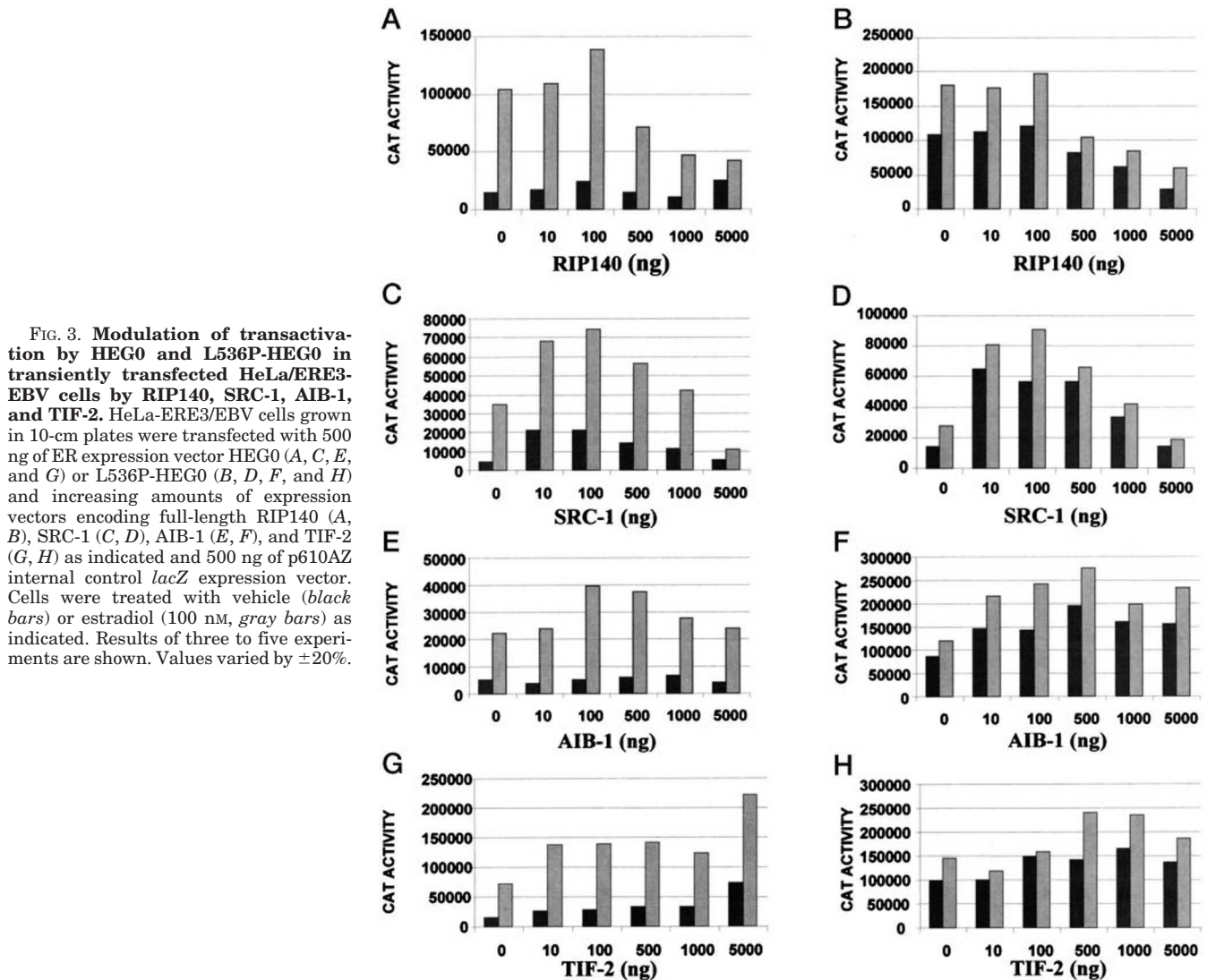


FIG. 3. Modulation of transactivation by HEG0 and L536P-HEG0 in transiently transfected HeLa/ERE3-EBV cells by RIP140, SRC-1, AIB-1, and TIF-2. HeLa-ERE3/EBV cells grown in 10-cm plates were transfected with 500 ng of ER expression vector HEG0 (A, C, E, and G) or L536P-HEG0 (B, D, F, and H) and increasing amounts of expression vectors encoding full-length RIP140 (A, B), SRC-1 (C, D), AIB-1 (E, F), and TIF-2 (G, H) as indicated and 500 ng of p610AZ internal control *lacZ* expression vector. Cells were treated with vehicle (black bars) or estradiol (100 nM, gray bars) as indicated. Results of three to five experiments are shown. Values varied by $\pm 20\%$.

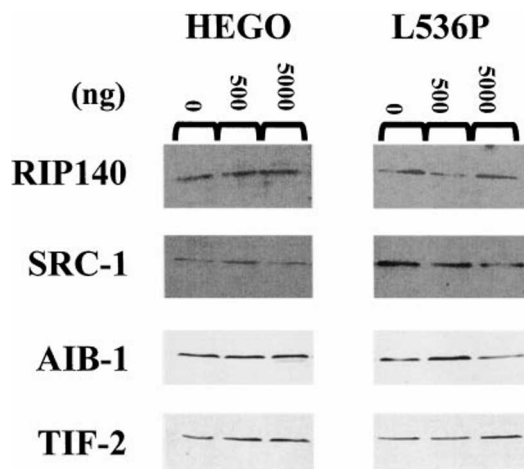


FIG. 4. Coexpression of coactivators does not affect estrogen receptor expression levels. Control Western blotting experiments were performed with extracts of transiently transfected HeLa/ERE-EBV cells used in Fig. 3. Amounts of coactivator expression vectors used in transfections are indicated. Blots were probed with the anti-ER B10 antibody as described previously (26).

high levels of transfected expression vector (5,000 ng) stimulated basal and hormone-stimulated transcription by HEG0 (Fig. 7). In contrast, cotransfection of 500 ng of RIP140 expres-

sion vector with 500 ng of HEG0 led to a modest inhibition of hormone-dependent transactivation by the ER. Stronger inhibitory effects were observed in the presence of higher concentrations of cotransfected RIP140 (data not shown). Moreover, addition of 500 ng of RIP140 expression vector completely abolished the stimulatory effect on ER-dependent transactivation of cotransfection of 5,000 ng of TIF-2 expression vector (Fig. 7). These results support the notion that coactivators may compete with each other *in vivo* (Figs. 5 and 6) and raise the possibility that RIP140 may serve a negative regulatory function.

DISCUSSION

Growing evidence supports the notion that coactivators would influence transcriptional initiation by different mechanisms by recruiting distinct sets of factors to preinitiation complexes. For example, CBP interacts selectively with coactivators of the SRC-1 family (13). SRC-1, AIB-1, and TIF-2 stimulate hormone-dependent transactivation by nuclear receptors in transient transfection assays, whereas RIP140 and TIF-1 function poorly or not all in these experiments (12, 18, 19, 21). This suggests that the final composition of preinitiation complexes may depend on the composition of coactivators present in a given cell. Our experiments indicate that an excess of one coactivator can compete for the binding of heterologous coactivators to the ER ligand binding domain *in vitro*. These exper-

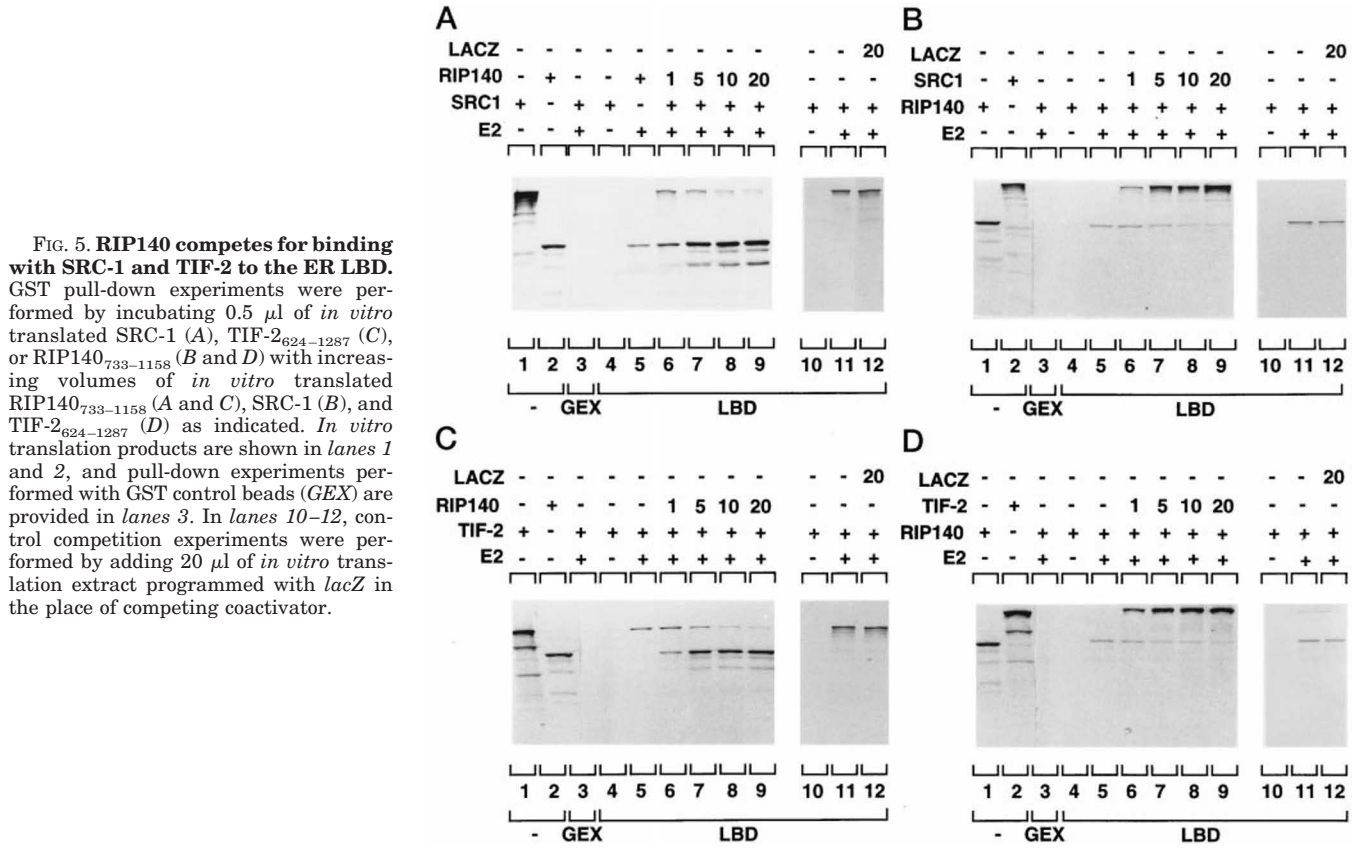


FIG. 5. RIP140 competes for binding with SRC-1 and TIF-2 to the ER LBD. GST pull-down experiments were performed by incubating 0.5 μ l of *in vitro* translated SRC-1 (A), TIF-2₆₂₄₋₁₂₈₇ (C), or RIP140₇₃₃₋₁₁₅₈ (B and D) with increasing volumes of *in vitro* translated RIP140₇₃₃₋₁₁₅₈ (A and C), SRC-1 (B), and TIF-2₆₂₄₋₁₂₈₇ (D) as indicated. *In vitro* translation products are shown in lanes 1 and 2, and pull-down experiments performed with GST control beads (GEX) are provided in lanes 3. In lanes 10-12, control competition experiments were performed by adding 20 μ l of *in vitro* translation extract programmed with *lacZ* in the place of competing coactivator.

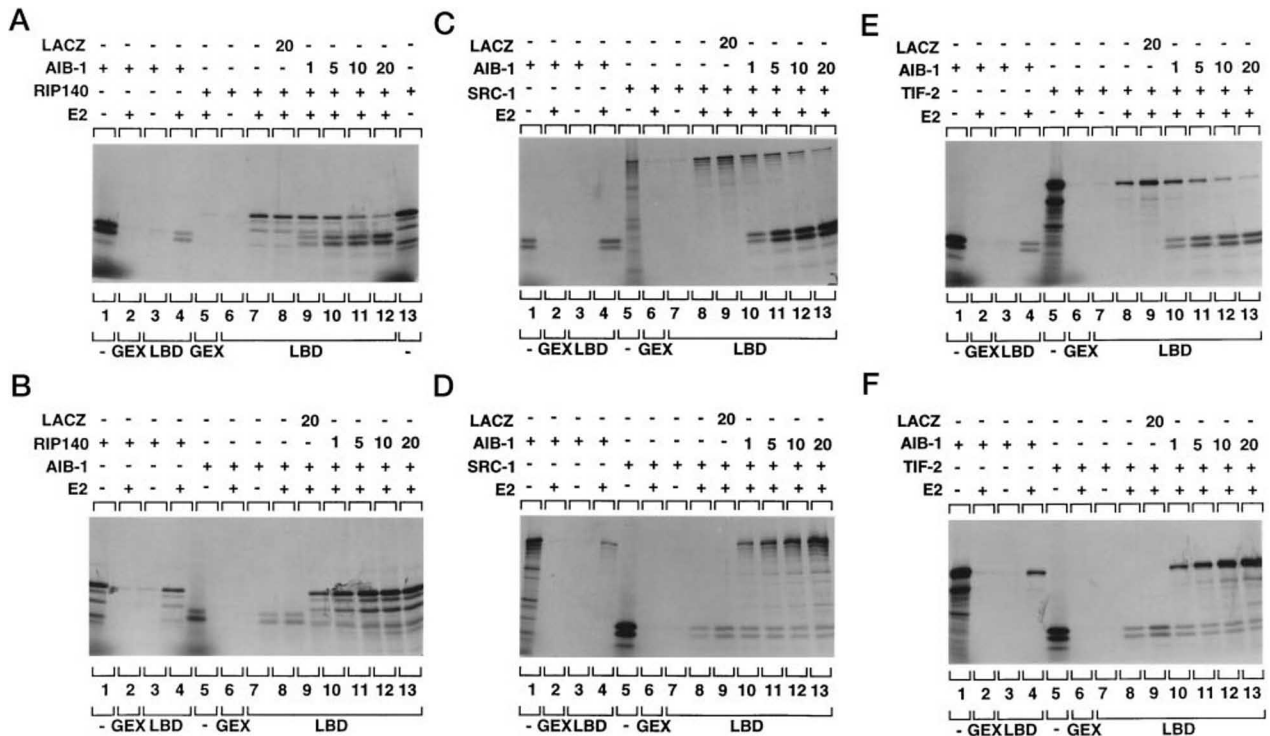


FIG. 6. Competitive binding of AIB-1 to the ER LBD. GST pull-down experiments were performed by incubating 0.5 μ l of *in vitro* translated RIP140₇₃₃₋₁₁₅₈ (A), SRC-1 (C), TIF-2₆₂₄₋₁₂₈₇ (E), or AIB-1₅₁₃₋₈₁₃ (B, D, and F) with increasing amounts of *in vitro* translated AIB-1₅₁₃₋₈₁₃ (A, C, and E), RIP140₇₃₃₋₁₁₅₈ (B), SRC-1 (D), or TIF-2₆₂₄₋₁₂₈₇ (F) as indicated. *In vitro* translation products are shown in lanes 1 and 13 (A, C, and E), lanes 1 and 5 (B-F), and pull-down experiments performed with GST control beads (GEX) are provided in lanes 2 and 5 (A) or 2 and 6 (B-F). In lanes 8 (A) or 9 (B-F) control competition experiments were performed by adding 20 μ l of an *in vitro* translation extract programmed with *lacZ* instead of competing competitor.

iments cannot rule out the possibility that coactivators present at similar concentrations may occupy simultaneously each of the LBDs of a DNA-bound ER homodimer. However, our re-

sults strongly suggest that interaction with the ER LBD is not stabilized by heterodimerization between different classes of coactivators, as no evidence was found that binding of a given

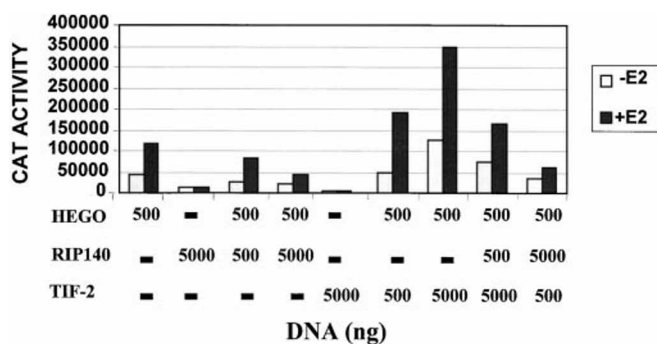


FIG. 7. RIP140 competes for TIF-2 function in HeLa-ERE3/EBV cells. HeLa-ERE3/EBV cells grown in 10-cm plates were transfected with an HEG0 expression vector and expression vectors for RIP140 and TIF-2 alone or in combination as indicated. Transfections were performed as described for Fig. 3.

coactivator to the ER LBD was enhanced by addition of a heterologous coactivator.

The observation that expression of RIP140 blocked the stimulatory effect of TIF-2 on ER function (Fig. 7) is consistent with GST pull-down assays performed with the receptor LBD and supported the notion that different classes of coactivators can modulate ER function in a competitive manner *in vivo*. These results are significant because TIF-2 has been shown to function by interacting with CBP, whereas RIP140 apparently does not (29). These results suggest that RIP140 may exert a negative regulatory effect on ER function under some conditions. We cannot rule out the possibility that the inhibitory effect of RIP140 may be due to squelching via indirect competition for basal transcription factors. However, it is important to note that, over the course of several experiments, cotransfection of increasing amounts of RIP140 expression vector up to 5,000 ng had no effect of expression of β -galactosidase activity from a heterologous promoter (data not shown). This suggests that the inhibitory effects of RIP140 are specific to the ERE-containing promoter in these experiments.

We conclude that different classes of coactivators recognize distinct but overlapping portions of the ER LBD. SRC-1, AIB-1, and RIP140 bound constitutively to the ER mutant L536P-HEG0, whereas TIF-2 and TIF-1 bound weakly in the absence of hormone (Fig. 3). Unlike other coactivators, TIF-1 bound to L536P-HEG0 in the presence of hormone at higher levels than to the wild-type ER LBD. In addition, RIP140, but not other coactivators, interacted with the wild-type ER LBD in the presence of the anti-estrogen ICI164,384, raising the possibility that, if RIP140 can function as an activator of ER-dependent transactivation, ICI164,384 may act like an estrogen agonist under specific conditions. These results are intriguing because they indicate that it is possible to design estrogenic compounds that would stimulate binding of specific coactivators or subsets of coactivators to the ER ligand binding domain and, thus, stimulate transactivation by the ER under specific metabolic conditions or in specific cell types. It will be important to fully characterize the different classes of coactivators expressed in specific estrogen target organs such as breast, endometrium, bone, and brain so as to understand better the molecular basis of the action of synthetic estrogenic or anti-estrogenic compounds.

Recently, it was found that expression of the coactivator AIB-1, which is related to SRC-1, was elevated in a number of hormone-dependent breast and ovarian cancer cell lines (15). The results suggested that AIB-1 (and other members of the SRC-1 family) is an important downstream component of ER-dependent signal transduction pathways and may mediate some of the mitogenic effects of estrogen in these cells. Signif-

icantly, we found that L536P-HEG0 functions constitutively in transiently transfected ER-positive MCF-7 breast carcinoma cells, which express high levels of AIB-1 (15). Moreover, the L536P-HEG0 LBD interacts constitutively with AIB-1 *in vitro*, and cotransfection of AIB-1 can stimulate basal transactivation by L536P-HEG0. These results suggest that mutations in the ER that cause hormone-independent binding of AIB-1 or other SRC-1 family members would lead to estrogen-independent proliferation of breast and ovarian tumors. This effect would be further enhanced in cells overexpressing AIB-1 or related coactivators. It is noteworthy that a mutation immediately adjacent to L536P, Y537N, which also confers constitutive activity on the ER, was recently identified in a metastatic breast tumor (30).

In summary, we have shown that different classes of coactivators can compete for binding to the ER ligand binding domain by recognizing distinct but overlapping regions of the LBD. We have shown that AIB-1 and SRC-1 interact constitutively with the LBD of the ER mutant L536P, consistent with the notion that acquisition of such mutations in the ER LBD *in vivo* would enhance hormone-independent proliferation of breast and ovarian tumors.

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