# Different Classes of Coactivators Recognize Distinct but Overlapping Binding Sites on the Estrogen Receptor Ligand Binding Domain <br> Frank C.S. Eng, Annie Barsalou, Naotake Akutsu, Isabelle Mercier, Christina Zechel, Sylvie Mader, John White 

## To cite this version:

Frank C.S. Eng, Annie Barsalou, Naotake Akutsu, Isabelle Mercier, Christina Zechel, et al.. Different Classes of Coactivators Recognize Distinct but Overlapping Binding Sites on the Estrogen Receptor Ligand Binding Domain. Journal of Biological Chemistry, 1998, 273 (43), pp.28371-28377. 10.1074/jbc.273.43.28371 . hal-04016634

HAL Id: hal-04016634
https://hal.science/hal-04016634
Submitted on 6 Mar 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

(Received for publication, January 23, 1998, and in revised form, July 15, 1998)

Frank C. S. Eng $\ddagger \S, ~ A n n i e ~ B a r s a l o u \eta, ~ N a o t a k e ~ A k u t s u ~ \# \|, ~ I s a b e l l e ~ M e r c i e r ॥, ~ C h r i s t i n a ~ Z e c h e l * *, ~$ Sylvie Mader $\|^{\ddagger} \ddagger$, and John H. White $\ddagger \S \S$<br>From the $\ddagger$ Departments of Physiology and Medicine, McGill University, Montréal H3G 1Y6, Canada, the 9Département de Biochimie, Université de Montréal, Montréal, Canada, and the **Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France

We have analyzed interaction of coactivators with the wild-type estrogen receptor $\alpha$ (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 can-cer-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.

[^0]The estrogen receptor $(E R)^{1}$ is a member of the family of nuclear receptors (1-6). Similar to other nuclear receptors, the $E R$ 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 (1216), TIF-2/GRIP-1 (17, 18), RIP140 (19), TIF- $1 \alpha$ and $-\beta(20,21)$, all of which apparently interact with receptors through signature motifs containing the core $\operatorname{LXXLL}(14,21,22)$. 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 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-

[^1]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 twohybrid 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 $\alpha$ 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 $15 \mu \mathrm{~g}$ 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 $250 \mu \mathrm{~g} / \mathrm{ml}$. 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 $100 \mu \mathrm{~g} / \mathrm{ml}$ 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 $1 \mu \mathrm{~g}$ of either HEG0/pSG5 or L536P-HEG0/ pSG5, $2 \mu \mathrm{~g}$ of ERE3-TATA-CAT/EBV reporter plasmid, and $2 \mu \mathrm{~g}$ of RSV-lac $Z$ as an internal control for transfection efficiency and $15 \mu \mathrm{~g}$ of Bluescript (Stratagene) carrier DNA. HeLa/ERE-EBV cells were transiently transfected by calcium phosphate coprecipitation with $1 \mu \mathrm{~g}$ of either HEG0/pSG5 or L536P-HEG0/pSG5, $2 \mu \mathrm{~g}$ of RSV-lacZ internal control, pSG5-based coactivator expression vectors added as indicated in the figures, and Bluescript carrier DNA to $20 \mu \mathrm{~g}$. 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 $\beta$-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 EcoRI 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 $37^{\circ} \mathrm{C}$ to an $A_{595}$ of 0.5. GST-ER expression was induced for 3 h at $30^{\circ} \mathrm{C}$ with 0.5 m isopropyl $\beta$-D-thiogalactopyranoside. Cells were centrifuged, and the bacterial pellet was resuspended in lysis buffer ( 50 mm Tris- $\mathrm{HCl}, \mathrm{pH}$ $7.5,100 \mathrm{~mm} \mathrm{NaCl}, 10 \mu \mathrm{M} \mathrm{ZnCl}{ }_{2}, 0.1 \mathrm{~mm}$ dithiothreitol, $10 \%$ glycerol, 100 $\mathrm{mg} / \mathrm{ml}$ 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 $\left[{ }^{35} \mathrm{~S}\right]$ methionine according to the manufacturer's instructions. Translation efficiency was determined by running 0.5 $\mu \mathrm{l}$ 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 $200 \mu \mathrm{l}$ of beads overnight at $4^{\circ} \mathrm{C}$. After three washes with GST buffer ( 50 mm Tris- $\mathrm{HCl}, \mathrm{pH} 7.5$, $100 \mathrm{~mm} \mathrm{NaCl}, 10 \mathrm{~mm} \mathrm{MgCl} 2,0.3 \mathrm{~mm}$ 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 $4^{\circ} \mathrm{C}$ for 2 h . The beads were then washed three times with $800 \mu \mathrm{l}$ 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, $10 \%$ ( $\mathrm{v} / \mathrm{v}$ ) acetic acid for 1 h , treated with $\mathrm{EN}^{3} \mathrm{HANCE}$ (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 L536PHEG0 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-HEGO - 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 es-trogen-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 es-trogen-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 $\pm 20 \%$.

A


C


B


D


L536P-HEG0 LBD was probed further by performing GST pulldown 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 $\left[{ }^{35} \mathrm{~S}\right]$ methionine. All coactivators, SRC-1, AIB- $1_{518-813}$, RIP $140_{733-1158}$, 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. $2 E$, 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 wildtype receptor (Fig. 2E, lane 8). Consistent with gene transfer experiments, OHT repressed coactivator binding to L536PHEG0 (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 2-3fold (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 hormoneindependent 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 hormonedependent 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 $L B D$-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 $10 \mu \mathrm{l}$ of GST-HEG0 LBD beads ( $2.5 \mu \mathrm{~g}$ 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 wildtype ER (HEGO) or the mutant L536PHEG0 (L536P) and in vitro translated RIP140 ${ }_{733-1158}(A)$, SRC-1 (B), AIB-1 $1_{518-}$ $813(C)$, 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 $(O H T)$, or 100 nm ICI164,384 (ICI) as indicated. An aliquot $(0.5 \mu \mathrm{l})$ of in vitro translation product is shown in lane 1 (lane 2 in $B$ ).

shown). Based on these experiments, 64 -fold dilutions of GSTHEG0 ( $0.039 \mu \mathrm{~g}$ 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 $0.5 \mu \mathrm{l}$ of in vitro translated coactivator, and increasing volumes ( 1 to $20 \mu \mathrm{l}$ ) 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, $20 \mu \mathrm{l}$ 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 $^{\text {(Fig. 5, } C \text { and }}$ $D)$. In another set of experiments, increasing amounts of AIB-$1_{518-813}$ competed for binding of RIP140 $0_{733-1158}$ (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/ERE3EBV cells by RIP140, SRC-1, AIB-1, and TIF-2. HeLa-ERE3/EBV cells grown in $10-\mathrm{cm}$ 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/EREEBV 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 ( $5,000 \mathrm{ng}$ ) 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 $5,000 \mathrm{ng}$ 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 $0.5 \mu \mathrm{l}$ of in vitro translated SRC-1 (A), TIF- $2_{624-1287}(C)$, or RIP140 ${ }_{733-1158}(B$ and $D$ ) with increasing volumes of in vitro translated RIP140 ${ }_{733-1158}(A$ and $C), S R C-1(B)$, and TIF- $2_{624-1287}(D)$ 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 $20 \mu \mathrm{l}$ of in vitro translation extract programmed with lac $Z$ 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 $0.5 \mu \mathrm{l}$ of in vitro translated RIP140 ${ }_{733-1158}(A)$, SRC-1 $(C)$, TIF- $2_{624-1287}(E)$, or AIB- $1_{513-813}\left(B, D\right.$, and $F$ ) with increasing amounts of in vitro translated AIB- $1_{513-813}$ (A, $C$, and $E)$, RIP140 ${ }_{733-1158}(B)$, SRC-1 $(D)$, or TIF- $2_{624-1287}(F)$ as indicated. In vitro translation products are shown in lanes 1 and $13(A)$ or lanes 1 and 5 $(B-F)$, and pull-down experiments performed with GST control beads $(G E X)$ 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 $20 \mu \mathrm{l}$ 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 $10-\mathrm{cm}$ 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 $5,000 \mathrm{ng}$ had no effect of expression of $\beta$-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 L536PHEG0, 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 antiestrogenic 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 ERdependent 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.

Acknowledgment-We thank Pr. Pierre Chambon (Illkirch, France) for the gift of coactivator expression vectors.

## REFERENCES

1. Beato, M., Herrlich, P., and Schutz, G. (1995) Cell 83, 851-857
2. Forman, B. M., Tontonoz, P., Chen, J., Brun, R., Spiegelman, B. M., and Evans, R. M. (1995) Cell 83, 803-812
3. Gronemeyer, H. (1991) Annu. Rev. Genet. 25, 89-123
4. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835-839
5. Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.-M., Argos, P., and Chambon, P. (1986) Nature 320, 134-139
6. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) Cell 59, 477-487
7. Montminy, M. (1997) Nature 687, 654-655
8. Wolffe, A. P. (1996) Science 272, 371-372
9. Wolffe, A. P., and Pruss, D. (1996) Cell 84, 817-819
10. Zawel, L., and Reinberg, D. (1995) Annu. Rev. Biochem. 64, 533-561
11. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) Mol. Endocrinol. 10, 1167-1177
12. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. (1995) Science 270, 1354-1357
13. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R., Rose, D. W., Glass, C. W., and Rosenfeld, M. G. (1996) Cell 85, 403-414
14. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. W., and Rosenfeld, M. G. (1997) Nature 387, 677-684
15. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X.-Y., Sauter, G., Kallioniemi, O.-P., Trent, J. M., and Meltzer, P. S. (1997) Science 277, 965-968
16. Li, H., Gomes, P. J., and Chen, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8479-8484
17. Hong, H., Kohli, K., Trivedi, A. Johnson, D. L., and Stallcup, M. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4948-4952
18. Voegel, J. J., Heine, M. J. S., Zechel, C., and Chambon, P. (1996) EMBO J. 15, 3667-3675
19. Cavaillès, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) EMBO J. 14, 3741-3751
20. LeDouarin, B., Zechel, C., Garnier, J.-M., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995) EMBO J. 14, 2020-2033
21. LeDouarin, B., Nielson, A. L., Garnier, J.-M., Ichinose, H., Jeanmougin, F., Losson, R., and Chambon, P. (1996) EMBO J. 15, 6701-6715
22. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) Nature 387, 733-736
23. Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Jugulon, H., Montminy, M., and Evans, R. M. (1996) Nature 383, 99-103
24. Ogryzko, V. V., Schiltz, R. I., Russanova, V., Howard, B., and Nakatani, Y. (1996) Cell 87, 953-959
25. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7879-7884
26. Eng F. C. S., Lee, H. S., Ferrara, J., Wilson, T. M., and White, J. H. (1997) Mol. Cell. Biol. 17, 4644-4653
27. White, J. H., McCuaig, K. A., and Mader, S. (1994) Bio/Technology 12, 1003-1007
28. Mader, S., and White, J. H. (1993) Proc. Nat. Acad. Sci. U. S. A. 90, 5603-5607
29. Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) EMBO J. 17, 507-519
30. Zhang, Q. X., Borg. A., Wolf, D. M., Oesterreich, S., and Fuqua, S. A. W. (1997) Cancer Res. 57, 1244-1249

[^0]:    * This work was supported by Grants MT-11704 (to J. H. W.) and MT-13147 (to S. M.) from the Medical Research Council of Canada and by grants from the Fonds pour la Formation des Chercheur et l'Aide à la Recherche (to S. M. and J. H. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
    § Recipient of a studentship from the Natural Sciences and Engineering Research Council of Canada.
    \| Recipient of a postdoctoral fellowship from the Royal Victoria Hospital Research Institute.

    护 Chercheur Boursier of the Fonds de Recherche en Santé du Québec.
    $\S \S$ Chercheur Boursier of the Fonds de Recherche en Santé du Québec. To whom correspondence should be addressed: Dept. of Physiology, McGill University, McIntyre Medical Sciences Bldg., 3655 Drummond St., Montréal H3G 1Y6, Canada. Tel.: 514-398-8498; Fax: 514-398-7452; E-mail: jwhite@physio.mcgill.ca.

[^1]:    ${ }^{1}$ The abbreviations used are: ER, estrogen receptor $\alpha$; 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 fac-tor-1; TIF-2, transcriptional intermediary factor-2; EBV, Epstein-Barr virus; CBP, CREB binding protein; CAT, chloramphenicol acetyltransferase; OHT, hydroxytamoxifen.

