Structure−Function Evaluation of ER α and β Interplay with SRC Family Coactivators. ER Selective Ligands

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ABSTRACT: Analysis of estrogen receptor α and β interplay with other transcription factors is critical to the understanding of how small molecules, the cognate ligands for these receptors, selectively regulate the mode and amplitude of gene transcription by affecting receptor activity. To better understand the molecular mechanisms of selective action of estrogen receptor ligands, we characterized estrogen receptor α and β (ER) interaction with the p160 family of coactivators. We also investigated how these interactions are affected by binding of specific ligands. We show that ER α and β utilize different LXXLL motifs for their interaction with p160 family members. We found that significant differences exist between the affinity of the nuclear receptor interacting domain (NRID) and interaction of separate LXXLL motifs with ERs. This result indicates that a single LXXLL motif is unlikely to be sufficient for interaction with receptors, and that regions other than LXXLL motifs also participate in ER−p160 complex formation. We found that ER α and β have strong affinity preferences for particular coactivators. These results suggest that ER-mediated transcription is not driven by a random mixture of ER−coactivator complexes. We also show that some ER ligands are functionally specific. We describe a ligand that binds to both receptors, but enhances only ER β interaction with SRC1 and SRC3 while exhibiting little effect on the ER α interaction with these proteins. Finally, we provide data that suggest how genistein may selectively recruit coactivators when liganded to ERs. It enhances the interaction of ERs with SRC1 and SRC3, but demonstrates a minimal effect on receptor interaction with DRIP205 and CBP.

Steroid hormones are widely distributed small, lipophilic molecules that participate in intracellular communication and control a wide spectrum of developmental and physiological processes. Their effects are mediated by specific intracellular receptors, a family of proteins that are characterized by a high affinity for their corresponding hormones and an ability to discriminate between structurally related ligands (*1*).

Steroid hormone receptors contain two well-characterized transactivation functions (AFs) (*2*, *3*). The activity of AF1, in the N-terminal portion of the ER molecule, is regulated by phosphorylation in response to growth factors (*4*, *5*), and the activity of AF2, in the ligand-binding domain (LBD),[1] is regulated by hormone binding. These two activation domains may function independently or synergistically depending on the cell type and target promoter (*3*).

In common with other transcription factors, the estrogen receptor stimulates transcription by recruiting a preinitiation complex. Although direct interactions between the ER and basal transcription factors TFIIB (*6*), TATA-binding protein [TBP (*7*)], and the TBP-associated factor [$TAF_{II}30$ (*8*)] have been demonstrated in vitro, they were shown to be ligand-independent and unaffected by mutations in H12 of the ligand binding domain that abolished AF2 activity. The involvement of other regulatory factors in receptor signaling was first postulated when nuclear receptors (NR) were found to functionally cross-react (squelch) with each other and with other classes of transcription factors (*9*). Since then, biochemical and genetic approaches have been used to identify and clone nuclear receptor-associated proteins, now called coregulators.

Most coregulators are, by definition, rate-limiting for nuclear receptor activation and repression, but do not significantly alter basal levels of transcription. Recent data have indicated multiple modes of coactivator action, which include direct bridging of nuclear receptors (NR) with basal transcription factors and enzymatic modification of histones and other proteins. This functional diversity is further amplified by coregulators themselves being a molecular target for multiple signaling pathways, which enables integration of these pathways in NR-mediated gene expression.

Among the most widely studied coactivators are the p160 family members. They were initially identified biochemically as ligand-dependent, nuclear receptor-interacting coactivators. The corresponding genes were cloned and identified as members of a family of steroid receptor coactivating factors, termed SRC-1/NcoA-1, TIF2/GRIP1/NcoA-2, and pCIP/ACTR/AIB1/SRC3 (*10*−*16*). Multiple reports (*11*, *17*) indicate that p160 family members may play different roles in NR-mediated transcription.

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[1] Abbreviations: ER, estrogen receptor; NRID, nuclear receptor interacting domain; NR, nuclear receptors; LBD, ligand binding domain; SERMs, selective estrogen receptor modulators; SRC, steroid receptor coactivator; GRIP, glucocorticoid receptor interacting protein.

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The ligand-dependent recruitment of these coregulators to nuclear receptors requires allosteric alterations in the AF2 domain. Structural analysis of the ligand binding domains (LBDs) of several NRs suggests that the binding of a ligand results in a realignment of helix 12 (*18*, *19*). Importantly, binding of two antiestrogens, raloxifene and 4-hydroxytamoxifen, leads to misalignment of helix 12, which blocks AF2 activity.

Most coactivator proteins contain one or more copies of a helical LXXLL motif (in which L denotes leucine and X denotes any amino acid), called NR boxes, that are found to be repeated throughout the p160s (*10*, *11*, *17*, *20*, *21*) and also within proteins that act as AF2 repressors, such as RIP140 (*20*, *22*) and TIF1 (*23*). For example, GRIP1 and its human analogue TIF2 contain three separate NR boxes (I−III, respectively), localized in their nuclear receptor interacting domain (NRID), of which NR boxes II and III are the most important for ER binding (*10*, *21*). The length and orientation of these helical motifs are important for high-affinity interaction with the receptor's AF2 domain, where an interaction surface is created by helices 3−5 and 12 of the LBD (*24*). Several groups have reported that regions C-terminal from the NRID might also interact with the AF-1 domain of estrogen receptors (*25*, *26*). This question, however, remains controversial given that several other groups have failed to detect this interaction (*10*, *17*).

Recently, a number of estrogen receptor ligands were characterized that display tissue selective action. These compounds have been termed SERMs (selective estrogen receptor modulators) since they reveal both agonist or antagonist activity in a cell type and promoter specific manner. The question as to how these ligands accomplish tissue selective activity remains to be elucidated. Among possible explanations are tissue selective expression of ER α and β (*27*) and tissue selective expression or "activation" of some ER coactivators (*15*, *17*). The hypothesis is that these compounds can differentially modulate interactions between the estrogen receptors and their partner proteins.

One of the major goals of this laboratory's work is to investigate the contribution of coactivators in the tissue and/or cell specific action of some NR ligands. In a series of experiments, we have examined how ERs interact with the p160 family of coactivators and how these interactions can be manipulated by ER ligands. Our data indicate that there are multiple levels of selectivity in ER−coactivator interactions that may determine the differential pharmacology of ER ligands.

MATERIALS AND METHODS

Equipment and Reagents. The BIAcore 2000 system, CM 5 sensor chips (certified), Tween-20, and amine coupling and GST capture kits were obtained from BIAcore Inc. Purified recombinant human estrogen receptors α and β were obtained from PanVera Corp. The buffer used for all experiments was 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.2 mM EDTA, 0.005% Tween-20, 1 mM DTT, 17β-estradiol (E2), and 4-hydroxytamoxifen were obtained from Sigma. Compound WAY164397, raloxifene, and genistein were synthesized by the Wyeth-Ayerst Medicinal Chemistry group. ICI-182,780 was provided by Astra-Zeneca Pharmaceuticals. The 13-mer peptides corresponding to LXXLL motifs of the SRC1 (NR box 1, SGSGHKLVQLLTT; NR box 2, SGHKILHRLLQEG; and NR box 3, SGHQLLRYLLDKD) were synthesized on a solid support using Wang resin and purified on a reverse phase column using HPLC.

Cloning and Protein Expression. Regions encoding nuclear receptor interacting domains (NRIDs) and regions containing NRID and CBP interaction domains of the SRC family coactivators, corresponding to amino acids 613−773 and 613−992 for SRC1 and amino acids 601−762 and 601−1101 for the SRC3, were PCR amplified using primers designed for the specific sites. Regions of the CBP molecule encoding the NR interaction domain corresponding to amino acids 1−253 were also PCR amplified using primers designed for the specific sites. The inserts were subcloned into pGEX-5X-3 at the *Sma*I and *Xho*I sites. BL21 (DE3) cells were transformed with the plasmid that was obtained.

Site-Directed Mutagenesis. NR box mutants were generated with the Quick-change site-directed mutagenesis system (Stratagene). The sequences for all mutant constructs were confirmed by the dideoxynucleotide chain termination reaction using the T7 Sequenase protocol (U.S. Biochemical Corp.).

Protein Purification. BL21 (DE3) cells were transformed with plasmids for expression of the GST−SRC1 and GST−SRC3, short and long forms, GST−Grip1, GST−DRIP-205, and GST−CBP constructs. Cells were grown at 37 °C until the OD_{600} reached 0.4. After that, the temperature was set to 24 °C and incubation was continued until the cell suspension OD_{600} reached 0.6. Protein expression was induced with 1 mM IPTG. Cells were incubated at 24 °C for an additional 3 h and harvested by centrifugation at 5000 rpm in a Sorval SS-34 rotor for 20 min and washed with PBS buffer, containing 1 mM DTT, 1 mM EDTA, and protease inhibitors cocktail (Sigma). Cells were sonicated, and the homogenate was centrifuged for 1.5 h at 45 000 rpm in a T45 rotor (Beckman). The supernatant was applied on a glutathione−agarose column (Pierce). Bound GST-fused proteins were eluted with a 5 to 50 mM gradient of reduced glutathione in 20 mM sodium phosphate buffer (pH 7.5), 2 mM DTT, and 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. Purified proteins were at least 90−95% pure as assessed by visualization on a Coomassie-stained SDS gel.

FLAG-$SRC3_{601-1412}$ was subcloned into pFLAG-MAC (Kodak-IBI, Rochester, NY) and expressed in BL21 (DE3) cells as previously described. Cells were sonicated, and FLAG-$SRC3_{601-1412}$ was purified on an anti-FLAG-M2−agarose column followed by overnight dialysis against 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1 mM DTT, and 10% glycerol.

Preparation of the Sensor Chip. Protein immobilization was performed as previously described (*28*, *29*). Briefly, first anti-GST or anti-FLAG antibody was immobilized using the amine coupling kit, according to the instructions from the manufacturer. Anti-GST antibody (6000−8000 RU) was typically immobilized with injection of 35 μL of 35 μg/mL antibody, and 8000−10000 RU of the anti-FLAG antibody was immobilized with injection of 60 μL of 20 μg/mL antibody over the chip surface activated with *N*-hydroxysuccinimide and *N*-ethyl-*N*′-[3-(diethylamino)propyl]carbodiimide (EDC). Second, purified GST- or FLAG-fused coac-

tivators were immobilized using antibody−antigen interaction. Injection (50 μL) of the protein with a concentration of 0.2−0.25 mg/mL typically allows immobilization of 600−800 RU of coactivator.

Binding Assay and Data Analysis. In the BIAcore instrument, one of the interacting molecules is immobilized onto a solid phase, the chip surface, while the other is introduced in flow over the surface. When a soluble macromolecule binds to the immobilized one, it leads to an increase in the macromolecule concentration at the sensor surface, with a corresponding increase in the refractive index. SPR measures changes in the refractive index close to the chip surface. The level of binding, measured in arbitrary response units (RU), is recorded in real time, and the obtained data contained information about the kinetics of macromolecule interaction. A signal of 1000 RU corresponds to a surface concentration change of approximately 1 ng/mm^2. A linear relationship exists between the mass (concentration) of molecules bound to the surface and the observed refractive index change (*29*). This approach was previously used to characterize NR interactions with specific DNA and other protein partners (*16*, *28*, *30*). Each binding cycle was performed with a constant flow of buffer at a rate of 10 μL/min. ER samples were injected across the surface via a sample loop. Once the injection plug had passed the surface, the formed complex was washed with buffer for an additional 500−1000 s. At the end of injection, the surface was regenerated with one 10 μL injection of a 0.05% SDS solution. To regenerate the surface and remove the immobilized GST-conjugated coactivator, we used a 10 μL injection of 10 mM glycine at pH 2.0. All experiments were performed at 25 °C. Data were collected at 1 Hz, and analyzed using BIAEvaluation software 3.0 (BIAcore, Inc.) on a Compaq personal computer. This program uses a global fitting analysis method for the determination of rate and affinity constants of macromolecular interactions. Refractive index differences between solutions of the ER at different protein and ligand concentrations were adjusted using Sigma Plot 5.0 software. In addition, nonlinear regression analysis utilized in Prism software (GraphPad Inc.) was used to determine the EC_{50} of receptor binding to p160s.

Cell Culture and Transient Transfection Assays. COS-7 cells were maintained in modified Eagle's medium (MEM) (Life Technologies, Inc.) supplemented with 10% fetal calf serum (HyClone, Logan, UT). For mammalian two-hybrid assays, functional domains of estrogen receptors and SRC coactivators were subcloned into pM and pVP16 vectors by PCR amplification using oligonucleotides designed for the appropriate regions. Typically, 4×10^4 cells per well were plated in 24-well plates 16 h prior to transfection in Dulbecco's modified Eagles's medium without L-glutamine or phenol red supplemented with 10% charcoal-stripped serum. Reporter pGL-luciferase (100 ng), 100 ng of pM, and 100 ng of pVP16 vectors (Clonetech) along with 20 ng of pCH110 β-galactosidase internal control vector (Pharmacia Biotech) were introduced into cells by Lipofectamine (Life Technologies, Inc.). Four hours after the cells had been incubated with the DNA/Lipofectamine mixture, cells were washed once with phosphate-buffered saline and incubated with Dulbecco's modified Eagles's medium without L-glutamine or phenol red supplemented with 10% charcoal-stripped serum with or without the indicated hormone for an additional 36 h. Cells were harvested 24 h later, and luciferase activity was quantified using a luminomter. The β-galactosidase activity of the cell lysates was determined and was used to normalize the luciferase activity.

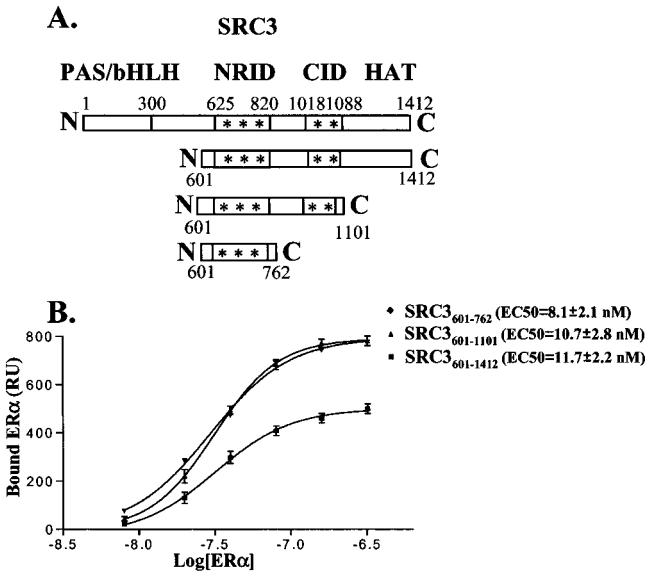

FIGURE 1: NRID is necessary and sufficient for interaction of estrogen receptors α and β with SRC3. (A) Derivatives of SRC3 used for interaction analysis. Asterisks show positions of the LXXLL domain. N-Terminal regions of SRC3 derivatives were fused to GST. (B) GST−SRC3 derivatives ($SRC3_{601-762}$, $SRC3_{601-1101}$, and FLAG-$SRC3_{601-1412}$, 280, 540, and 610 RU, respectively) were immobilized on a sensor chip surface using an anti-GST or anti-FLAG antibody. These proteins were then incubated with ER α in the presence of 17β-estradiol (1 μM). Concentrations of ER α varied from 1.12 to 112 nM. ER α was injected at a rate of 10 μL/min for 10 min (100 μL of protein). The amount of bound ER α (RU) is plotted vs receptor concentration. Nonlinear regression analysis utilized in Prism software (GraphPad Inc.) was used to determine the EC_{50} of receptor binding to the p160 derivatives.

RESULTS

The NRID Determines the Affinity of SRC Interaction with Estrogen Receptors. To determine if regions outside the NRID contribute to p160 interaction with estrogen receptors, we compared the affinity of ER α and β interaction with certain derivatives of SRC3 and SRC1 molecules (Figure 1A). To do so, we have utilized an approach called real time interaction analysis with the BIAcore instrument. We have previously used this method to characterize nuclear receptor interaction with other proteins and DNA (*28*−*30*). Derivatives of the SRC1 and SRC3 molecules (Figure 1A) were expressed as GST fusion proteins and purified to near homogeneity using glutathione−agarose beads and gel filtration on a Superdex 200 column.

GST−SRC3 Derivatives. $SRC3_{601-762}$, $SRC3_{601-1101}$, and FLAG-$SRC3_{601-1412}$ (280, 540, and 610 RU, respectively) were immobilized on a chip surface using an anti-GST or anti-FLAG antibody. These proteins were incubated with ER α or β in the presence of 17β-estradiol (1 μM). Concentrations of ER α varied from 1.12 to 112.0 nM. Figure 1B presents a plot of the amount of ER α (RU) bound to the SRC3 derivative at the end of each injection against the logarithm of the receptor concentration. The EC_{50} of receptor binding to all p160 derivatives was around 10 nM (see Figure

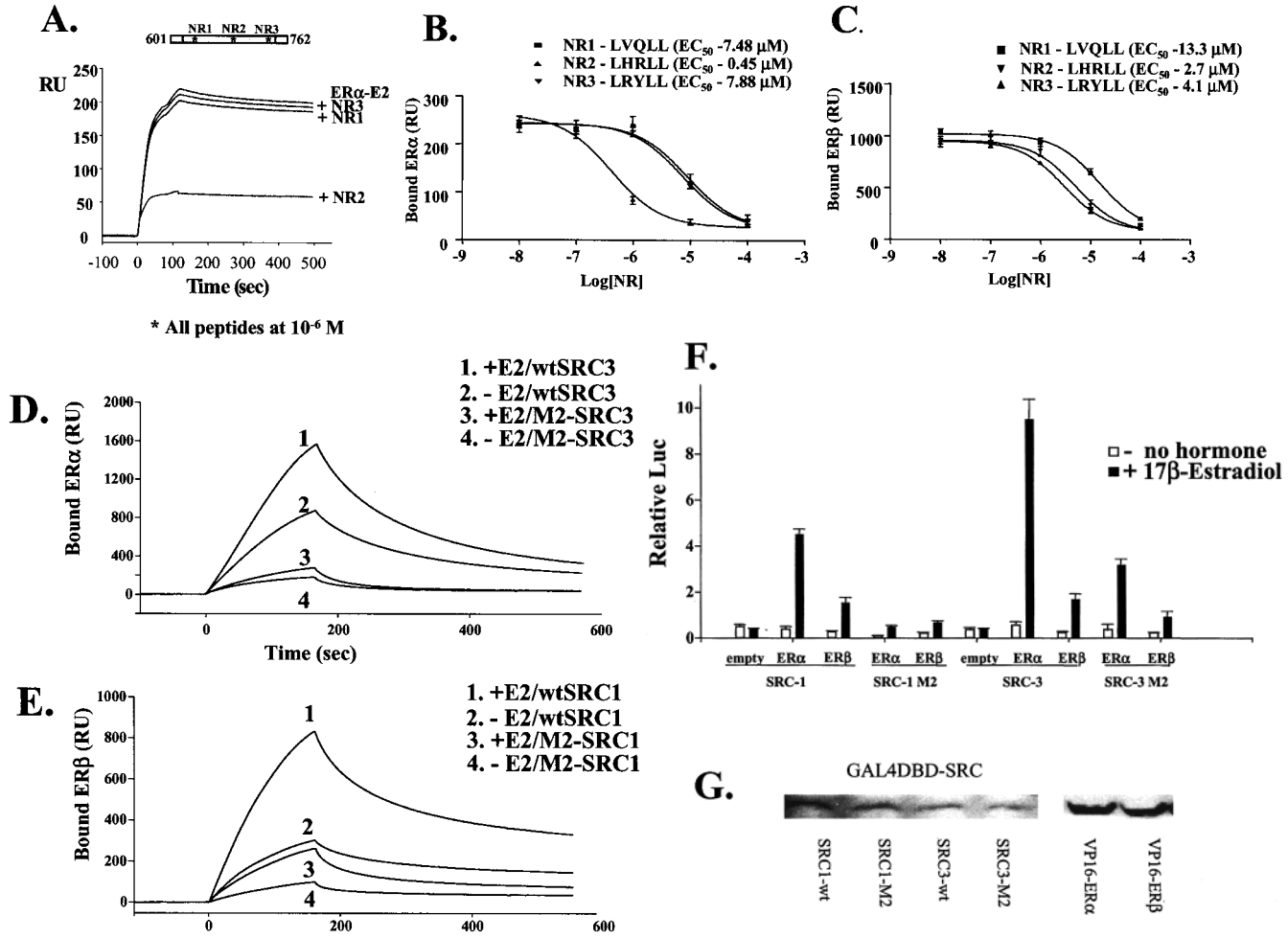

FIGURE 2: NR boxes play different roles in p160−ER complex formation. (A) Overlaid sensograms of the ER α injection at a protein concentration of 9 nM over the surface with immobilized $SRC3_{601-762}$ (280 RU). Before the injection, ER α was preincubated with 17β-estradiol and the corresponding peptide (both at 1 μM) for 1 h at room temperature. (B) ER α was preincubated with 17β-estradiol at 1.0 μM, and one of the NR boxes at concentrations ranging from 10 nM to 0.1 mM. The amount of ER α, bound to $SRC3_{601-762}$ at the end of each injection, is plotted vs the logarithm of the peptide concentration. (C) ER β was preincubated with 17β-estradiol at 1.0 μM and one of the NR boxes at concentrations ranging form 10 nM to 0.1 mM. The amount of ERα bound to $SRC1_{613-773}$ at the end of each injection is plotted vs the logarithm of the peptide concentration. (D and E) Wild-type and mutated SRC3 (870 RU, D) and SRC1 (820 RU, E) were immobilized on a surface of a sensor chip using an anti-GST antibody. Overlaid sensograms of injections of liganded with 17β-estradiol (injections 1 and 3) and the unliganded ER (injections 2 and 4) over the wild-type (injections 1 and 2) and mutated (injections 3 and 4) coactivators. The ER α concentration was 23 nM, and the ER β concentration was 62 nM. (F) Mammalian two-hybrid interaction analysis. COS-7 cells (2×10^4) were transfected with 100 ng of expression vectors for GAL4DBD fused to either the wild type or M2 mutant of $SRC1_{613-773}$ or $SRC3_{601-762}$. One hundred nanograms of expression vectors for VP16 fused to ER α (amino acids 241−596) or ER β LBD (amino acids 205−531) and 100 ng of UAS-Luc reporter and 20 ng of pCMV β-Gal internal control. Either ethanol vehicle (white bar) or 10^{-6} M 17β-estradiol (black bar) was added to cells for 36 h and then assayed for luciferase activity normalized to β-Gal control. The level of induction in the presence of 17β-estradiol is shown. (G) Western blotting analysis shows the relative expression levels of the bait and prey.

1B), which indicates that the contribution of the C-terminus to NRID portions of their molecules is not very significant, at least in a context of overexpressed and purified receptor and coactivator. Similar results were also obtained for ER α−SRC1 and ER β−SRC1 and −SRC3 interactions.

Role of the NR Boxes in p160−ER Complex Formation. The goal for the next set of experiments was to investigate how different NR boxes within the NRID domain contribute to p160−ER complex formation, and if peptides corresponding to different NR boxes can be used to control these interactions. To address this question, we used a competition assay with 14-mer peptides corresponding to different NR boxes. ER α (in the presence of 1 μM 17β-estradiol) was incubated with one of the peptides (final concentration of 1 μM) and injected over the surface with immobilized $SRC3_{601-762}$. A peptide corresponding to NR box 2 significantly reduced the amount of the ER α−E2 species bound to the SRC3−NRID (Figure 2A). At the same time, peptides corresponding to NR boxes 1 and 3 at 1 μM did not significantly alter ER α−E2 binding. To quantitatively assess the ability of the peptides corresponding to NR boxes 1−3 to bind to ER α and β in the presence of 1 μM 17β-estradiol, competition experiments were performed with peptide concentrations ranging from 10.0 nM to 0.1 mM. The amount of the ER α−E2 species (Figure 2B) bound to $SRC3_{601-762}$ or ER β (Figure 2C) bound to $SRC1_{613-992}$ at the end of each interaction cycle was plotted against the logarithm of the peptide concentration. These results indicate that all NR boxes can be utilized for ER−p160 complex formation. However, it appears that NR box 2 interacts with ER α with

Table 1: NR Boxes Bind to ER with Different Affinities (EC_{50} in micromolar)

ER α	NR box 1	NR box 2	NR box 3
SRC1	6.08 ± 1.8	0.45 ± 0.17	6.09 ± 2.4
SRC2	5.46 ± 3.1	0.41 ± 0.13	6.25 ± 3.4
SRC3	7.46 ± 3.4	0.45 ± 0.26	7.88 ± 3.5
ER β	NR box 1	NR box 2	NR box 3
SRC1	10.6 ± 2.3	4.82 ± 0.17	3.07 ± 1.1
SRC2	15.3 ± 1.3	3.63 ± 0.13	2.14 ± 0.7
SRC3	13.3 ± 1.6	5.74 ± 0.26	4.08 ± 1.6

a higher affinity than NR box 1 or 3. This is supported by the apparent EC_{50} of 0.45 μM for NR box 2, versus values of 7.5 and 7.9 μM for NR boxes 1 and 3, respectively. Interestingly, peptides corresponding to NR boxes 2 and 3 compete for ER β binding to $SRC1_{613-992}$ about equally well. Similar data were obtained in a context of ER α and ER β interaction with $SRC1_{613-992}$, $SRC3_{601-762}$, and $GRIP1_{618-766}$ (see Table 1). These results clearly indicate that ER α and β may utilize different LXXLL motifs for their interaction with p160s. Furthermore, separate LXXLL motifs interact with ERs with only micromolar affinity, while NRIDs interact with receptors with affinities in the nanomolar range (see Figure 1B). These results suggest that regions other than LXXLL motifs may participate in ER−p160 complex formation.

Crystallographic and mutational analyses have revealed the essential role of leucine residues in NR box interaction with nuclear receptors (*10*, *17*, *21*, *31*). To further address the question of whether NR boxes can substitute for each other in the ER−p160 interaction, we expressed and purified GST−NRID of SRC1 and SRC3 in which all three leucines in the NR box 2 were substituted with alanines.

Wild-type and mutated $SRC1_{NRID}$ and $SRC3_{NRID}$ were used in real time interaction analysis experiments to compare their interaction with ER α and β. Panels D and E of Figure 2 present sensograms of overlaid injections of the ER liganded with 17β-estradiol (1 μM) (injections 1 and 3) and the unliganded ER (injections 2 and 4) over the wild-type (injections 1 and 2) and mutated (injections 3 and 4) SRC1 and SRC3. These results indicate that mutation of NR box 2 significantly reduces but does not abolish ligand-dependent ER−p160 complex formation. Very similar data were obtained using mammalian two-hybrid interaction analysis (Figure 2F). These results indicate that NR boxes can substitute for each other, promoting ER−coactivator interaction.

Estrogen Receptors Manifest Different Affinities in Their Interaction with Coactivators. Ding et al. (*21*) have recently demonstrated that some NRs demonstrate preferences in their interaction with coactivators. AR, for example, binds preferentially to GRIP1 over SRC1. We have also previously shown that SRC3 interacts with ER α with a higher affinity than with ER β (*16*). We therefore decided to determine affinities of estrogen receptor interaction with members of the p160 family coactivators.

Immobilized derivatives of corresponding coactivators $SRC3_{601-762}$, $SRC1_{613-773}$, and $GRIP1_{618-766}$ were allowed to interact with estrogen receptors α and β in the presence of 17β-estradiol (1 μM), at increasing receptor concentrations ranging from 1.13 to 112.5 nM for ER α and from 3.13 to 312.0 nM for ER β. ER−coactivator complex formation was detected using real time interaction analysis. To evaluate affinity of receptor−coactivator interaction, we used global fitting analysis with BIAevaluation 3.1 software. This software uses numerical integration algorithms to calculate affinity and kinetic rates. Using this software, we evaluated several possible interaction models. The results do not fit adequately into a simple monomolecular interaction model ($A + B \leftrightarrow AB$). Instead, they fit well to a model that describes a two-state reaction ($A + B \leftrightarrow AB \leftrightarrow AB^*$). At present, we do not know the exact nature of this second step in ER−p160 complex formation. We speculate that it can be a conformational change that this complex undergoes after initial binding. Affinity rate constants are presented in Figure 3. These data indicate that estrogen receptors bind coactivators with different affinities and that SRC3 is the preferred partner exhibiting the highest-affinity interaction with ER α. ER β interacts with p160 family members with lower affinity, which may explain why its transcriptional activity is lower. These data also indicate that NR-driven transactivation is not mediated by a random set of receptor−coactivator complexes.

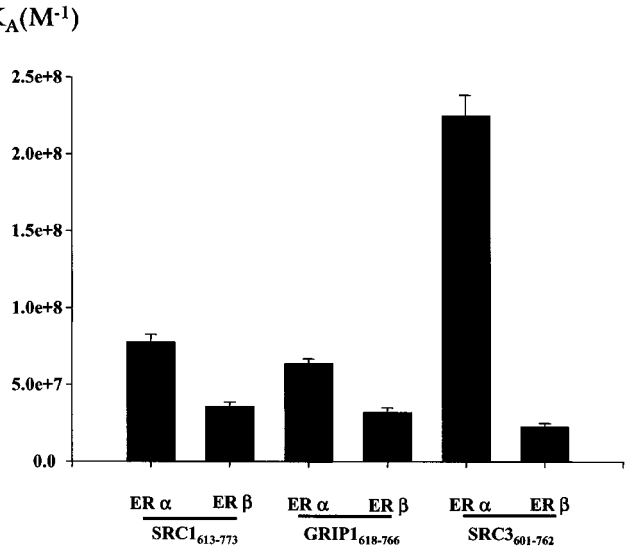

FIGURE 3: Estrogen receptors α and β exhibit affinity preferences for particular coactivators. Immobilized derivatives of corresponding coactivators ($SRC3_{601-762}$, $SRC1_{613-773}$, and $GRIP1_{618-766}$) were allowed to interact with estrogen receptors α and β in the presence of 17β-estradiol (1 μM) at increasing receptor concentrations ranging from 1.13 to 112.50 nM for E Rα and from 3.13 to 312 nM for ER β. Data were analyzed using BIAEvaluation version 3.0. K_A constants were calculated using a model that describes receptor binding and conformational readjustment of the ER−SRC complex.

Estrogen Receptors Bind Coactivators Ligand-Dependently. It has been demonstrated that NR agonists promote receptor interaction with coactivators and antagonists block this interaction. Structural analysis revealed the molecular nature of this phenomenon. Binding of ligand results in realignment of helix 12 (*32*−*35*). Its functional importance to the ER is indicated by the observation that it is misaligned in the presence of raloxifene (*36*) and 4-hydroxytamoxifen, which block AF2-mediated transcriptional activity.

A number of ER ligands have been characterized recently that are agonists or antagonists depending upon the cell type and promoter context. It has also been established that ER

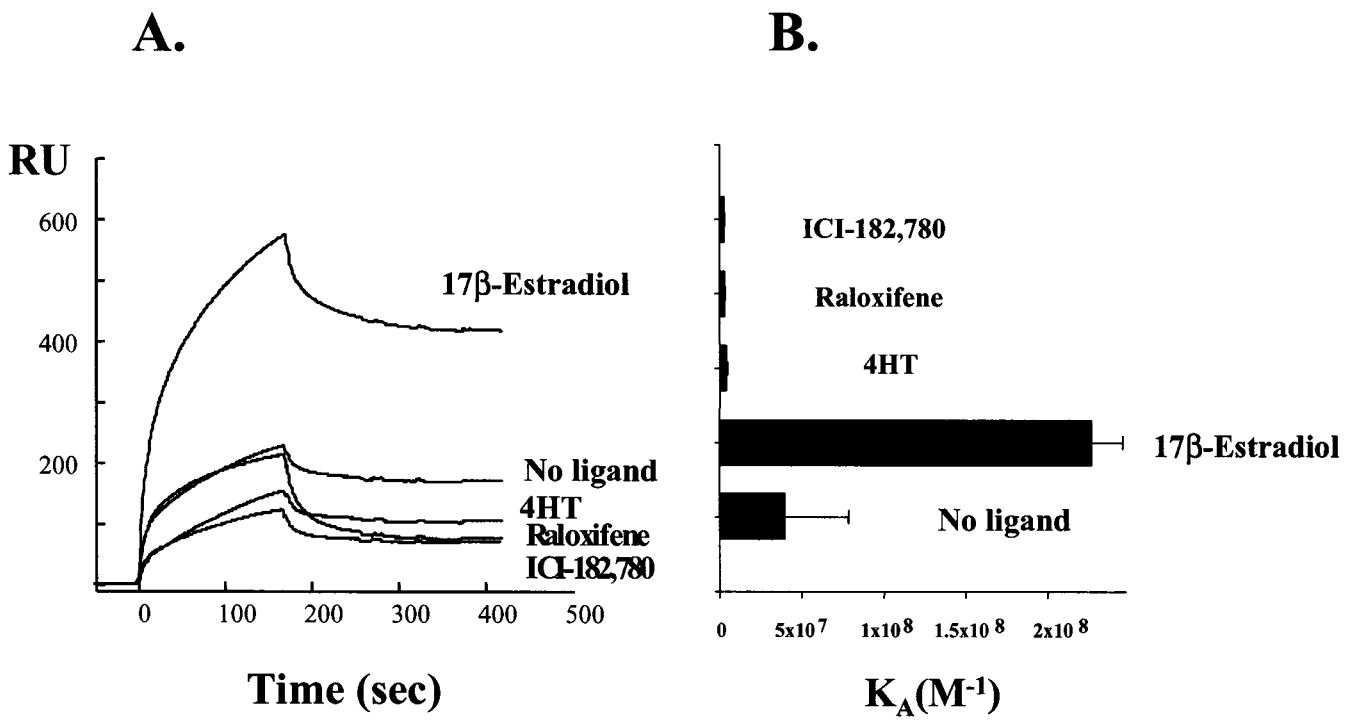

FIGURE 4: Ligands affect the affinity of interaction of ER α with SRC3. (A) Overlaid sensograms of ERα injections over the immobilized $SRC3_{601-762}$. Prior to the injection, the receptor (10 nM) was preincubated with the corresponding ligand (1 μM) for 1 h at room temperature. (B) Affinity constants for the ER α interaction with $SRC3_{601-762}$ in the presence of 17β-estradiol, 4-hydroxytamoxifen, raloxifene, or ICI-182,780 all at 1 μM were calculated as described in Materials and Methods.

ligands vary in their efficacy. A possible explanation is that these ligands induce different affinities in receptor−coactivator interactions. To better understand the molecular mechanism of a ligand's action, we have evaluated how the affinity of estrogen receptor interaction with p160 family members is affected by ligand binding.

Serial injections of unliganded ER α or β, or receptors liganded with E_2, 4-hydroxytamoxifen, raloxifene, and ICI-182,780, at protein concentrations ranging from 3.7 to 400 nM, were run over the sensor chip with immobilized coactivators. Figure 4A presents overlaid sensograms of injections of unliganded ER α, and ER α liganded with E_2, 4-hydroxytamoxifen, raloxifene, and ICI-182,780 run over immobilized SRC3 These results indicate that binding of 17β-estradiol enhances the affinity of the ER α−SRC3 interaction, compared to that of the unliganded receptor, and that ICI-182,780, 4-hydroxytamoxifen, and raloxifene all inhibit this interaction. Similar results were obtained for the interaction of ER α and β with SRC1, GRIP1, CBP, and DRP205 (data not shown). Affinity rate constants were calculated, as previously described, using global fitting analysis with BIAEvaluation 3.1 software and are summarized in Figure 4B.

These data suggest that conformational changes induced by ligand binding impose different affinities for receptor−coactivator interactions. Binding of 17β-estradiol increased the affinity of ER α interaction with SRC3 ~20-fold, compared to that of the unliganded receptor. At the same time, binding of 4-hydroxytamoxifen reduces the affinity of this interaction almost 3-fold.

Functionally Specific ER Ligands. The existence of two estrogen receptors, ER α and β, their differential expression (*27*), and considerable sequence dissimilarity has contributed to significant interest in development of receptor selective ligands. We tested a number of compounds for their effect on ER−coactivator interaction. For most of them, a good correlation was found between ligand binding and its effect on these interactions. For example, the EC_{50} for the 17β-estradiol effect on ER α and β binding to SRC1 and SRC3 was ~1 nM (data not shown) which correlates well with the EC_{50} of 17β-estradiol binding to ER α and β. However, for some compounds, significant discrepancies between these parameters were documented.

Compound WAY164397 competes for 17β-estradiol binding to ER β with an IC_{50} of 8 nM, and to ER α with an IC_{50} of 240 nM (data not shown). We asked what effect this compound has on ER α and β interaction with $SRC1_{NRID}$ and $SRC3_{NRID}$. To address this question, both receptors were preincubated with a saturating concentration of WAY164397 (5.0 μM) before they were injected over the surface containing an immobilized GST−$SRC1_{NRID}$ or GST−$SRC3_{NRID}$ compound. Figure 5A−D presents overlaid injections of ER α and β unliganded and liganded with 17β-estradiol, WAY164397, and ICI-182,780 over immobilized SRC1 and SRC3. These data indicate that WAY164397 augments the affinity of the ER β interaction with both SRC1 and SRC3 (see Figure 5A,B); however, even a saturating concentration has very little influence on ER α interaction with these proteins (Figure 5C,D). These results clearly indicate that nuclear receptor ligands can be functionally selective. They may bind to both ER α and β, but differentially affect the interaction of the receptors with coactivators.

Coactivator Specific Ligands. Tissue selective action of some estrogen receptor ligands may be mediated by selectively expressed and/or activated ER-interacting proteins (*13*, *17*). Therefore, compounds that selectively modulate ER interaction with coactivators may have important therapeutic activity. In the next experiment, we asked if ER ligands affect

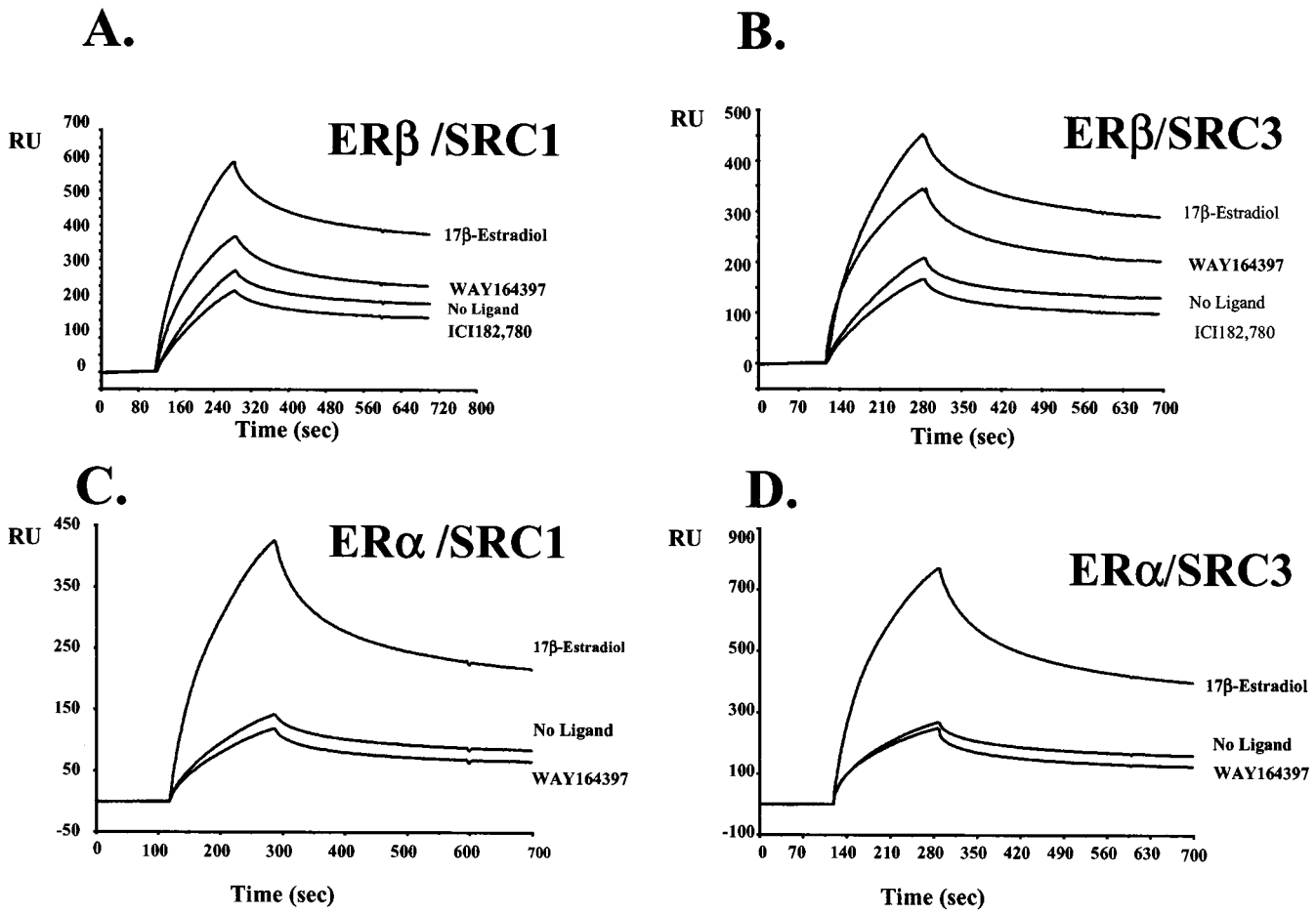

FIGURE 5: WAY164397 is an ER β selective ligand. (A) Overlaid sensograms of ER β injections over the immobilized $SRC1_{613-773}$ (608 RU) in the absence or presence of 1.0 μM 17β-estradiol, WAY164397, or ICI-182,780. The ER β concentration for all injections was 25 nM. (B) Overlaid sensograms of ER β injections over the immobilized $SRC3_{601-762}$ (623 RU) in the absence or presence of 1.0 μM 17β-estradiol, WAY164397, or ICI-182,780. The ER β concentration for all injections was 25 nM. (C) Overlaid sensograms of ER α injections over the immobilized $SRC1_{613-773}$ (608 RU) in the absence or presence of 17β-estradiol, WAY164397, or ICI-182,780, all at 1.0 μM. The ER β concentration for all injections was 10 nM. (D) Overlaid sensograms of ER α injections over the immobilized $SRC3_{601-762}$ (608 RU) in the absence or presence of 17β-estradiol, WAY164397, or ICI-182,780, all at 1 μM. The ER β concentration for all injections was 10 nM.

receptor binding to DRIP205, CBP, and p160 family members in the same way. Bacterially expressed and purified $GST-CBP_{1-253}$ and $GST-DRIP205_{527-970}$ species, in addition to GST−NRID complexes of p160 family members described previously, were used for this experiment. We found that 17β-estradiol enhanced affinity for interaction of the all studied coactivators with ERs, while 4-hydroxytamoxifen, raloxifene, and ICI-182,780 inhibited these interactions. Interestingly, genistein demonstrated coactivator selective activity. Figure 6A−D presents overlaid injections of unliganded ER α, and receptor liganded with 17β-estradiol and genistein over the immobilized SRC1, SRC3, CBP and DRIP205. The data indicate that genistein promotes ER α interaction with SRC1 and SRC3 (Figure 6C,D) but exerts a minimal effect on interaction with DRIP205 and CBP (Figure 6A,B). Similar results were also obtained for ER β (data not shown).

DISCUSSION

To understand the molecular nature of tissue, cell, and promoter selectivity of some estrogen receptor ligands, we need to learn how their binding affects receptor activity, ER binding to DNA, and interaction with other transcription factors. In this work, we have investigated ER−p160 interplay, and evaluated the ligand's role in their interaction. The p160 family is defined by an overall sequence similarity of 40% among its three family members. The most apparent is sequence conservation in their N-terminal domain. While considerable sequence similarity between p160 family members indicates some redundancy of function, there is sufficient sequence divergence to suggest functional autonomy. A recent comparison between SRC1, TIF2, and pCIP revealed that p160 proteins are functionally distinct. First, SRC1's role appears to be restricted to NRs, while pCIP is also required for other CBP/p300-dependent transcription factors such as STATs (*11*). Furthermore, SRC1 was shown to be essential for RAR signaling, but TIF2/GRIP is dispensable (*11*). In addition, SRC1 isoforms (a and e), which diverge at their C-termini, were shown to be functionally distinct, as they differ in their ability to interact and enhance ER-driven transactivation (*17*).

A common structural feature among p160 family members is the NR boxes, a recurrent pentapeptide motif that appears to direct the interaction of coactivators with their receptor partners. Three of these motifs are conserved in both sequence and spacing in all family members (*11*, *20*) and

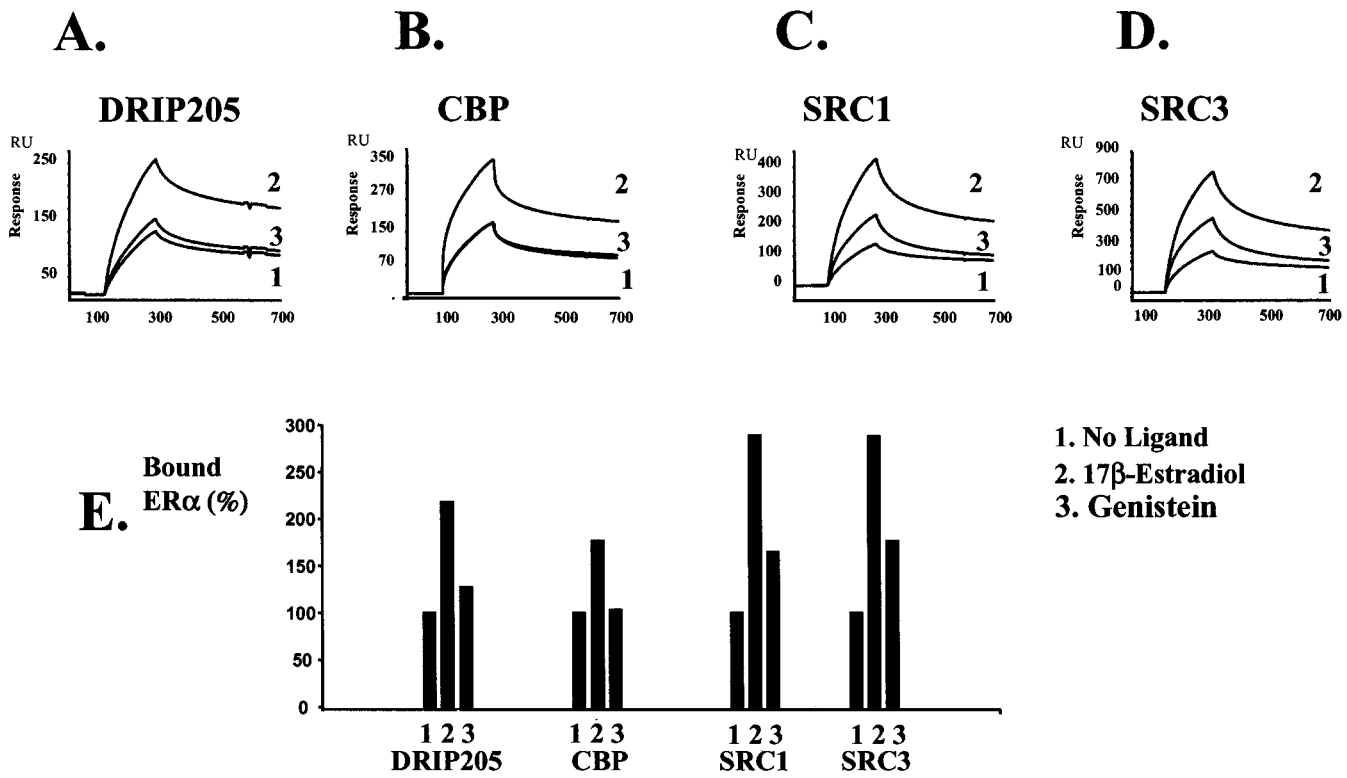

FIGURE 6: Genistein is a coactivator selective ligand. (A) Overlaid sensograms of ER α injections over the immobilized $DRIP205_{527-970}$ (560 RU) in the absence or presence of 17β-estradiol or genistein, either at 1 μM. The ER α concentration for all injections was 10 nM. (B) Overlaid sensograms of ER α injections over the immobilized CBP_{1-253} (630 RU) in the absence or presence of 17β-estradiol or genistein, either at 1 μM. The ER α concentration for all injections was 10 nM. (C) Overlaid sensograms of ER α injections over the immobilized $SRC1_{613-773}$ (520 RU) in the absence or presence of 17β-estradiol or genistein, either at 1 μM. The ER α concentration for all injections was 10 nM. (D) Overlaid sensograms of ER α injections over the immobilized $SRC3_{601-762}$ (540 RU) in the absence or presence of 17β-estradiol or genistein, either at 1 μM. The ER α concentration for all injections was 10 nM. (E) Summary of the binding data. The graph shows the amount of bound ER α at the end of each injection. The amount of unliganded ER α bound to the coactivator is set to 100%.

are localized in the central portion of their molecules, called the nuclear receptor interaction domain (NRID). It has been demonstrated that regions outside of the NRID can interact with ER and stabilize ER interaction with SRC1a. The role of this interaction is not clear since mutation of this motif has no effect on the activity of SRC1a in potentiating transcriptional activity of the ER in transiently transfected cells (*17*). It has been also suggested that regions outside of the NR interaction domain (NRID) may contribute to p160 interaction with nuclear receptors (*31*). Several groups have reported that regions C-terminal to the NRID might also interact with the AF-1 domain of estrogen receptors (*25*, *26*). However, this question remains controversial given that others have failed to detect this interaction (*10*, *17*). The domain that is C-terminal to the NRID has been shown to have additional NR boxes and interact with CBP/p300; when tethered to DNA, it can activate transcription (*10*, *17*).

We first asked what region(s) of the p160 molecule is responsible for the high-affinity interaction with estrogen receptors. To address this question, we created a number of p160 deletion mutants. These derivatives were expressed as GST fusion proteins, and their affinities for full-length ER α and β were evaluated using real time interaction analysis. Our data indicate that the affinity of the ER−p160 interaction is essentially determined by the corresponding NRID, and that regions outside of the NRID, including the CBP interaction domain, do not have a detectable impact on the affinity of the p160−ER interaction.

LXXLL motifs (NR boxes) have previously been shown to play a critical role in the p160 interaction with nuclear receptors (*6*, *20*, *37*). X-ray analysis has demonstrated that the second NR box of GRIP1 directly interacts with helices 3−5 and 12 of the ER α LBD (*36*, *38*). It has been suggested that distinct NR boxes are involved in p160 interaction with different nuclear receptors (*15*, *37*). We asked if a single NR box binding to the receptor determines ER−p160 interaction. We evaluated the affinity of the NR box binding to ERs. Our data indicate that NR box 2 binds to ER α with an EC_{50} of 0.5 μM. In contrast, NR boxes 1 and 3 bind 10 times less potently. Surprisingly, NR boxes 2 and 3 bind to ER β with very similar EC_{50}s of 2.7 and 4.1 μM, respectively. These data indicate that ER α and β may utilize different LXXLL motifs for their interaction with p160 proteins. Furthermore, separate LXXLL motifs interact with ERs with only micromolar affinity, while NRIDs interact with receptors with affinities in the nanomolar range. To evaluate if NR box 2 is an absolute prerequisite for the ER−p160 interaction, we mutated all leucines to alanines in NR box 2 of SRC1 and SRC3. Interactions of these mutants with ER α and β were studied using mammalian two-hybrid and real time interaction analysis. Our data indicate that NR box 2 mutations dramatically reduce, but do not abrogate, p160−ER interaction, which suggests that NR boxes can substitute for each other. It has been shown that mutations of the NR and NR boxes 1 and 3 (*17*) also reduce the extent of ER α−SRC1 interaction. These results, in conjunction with the

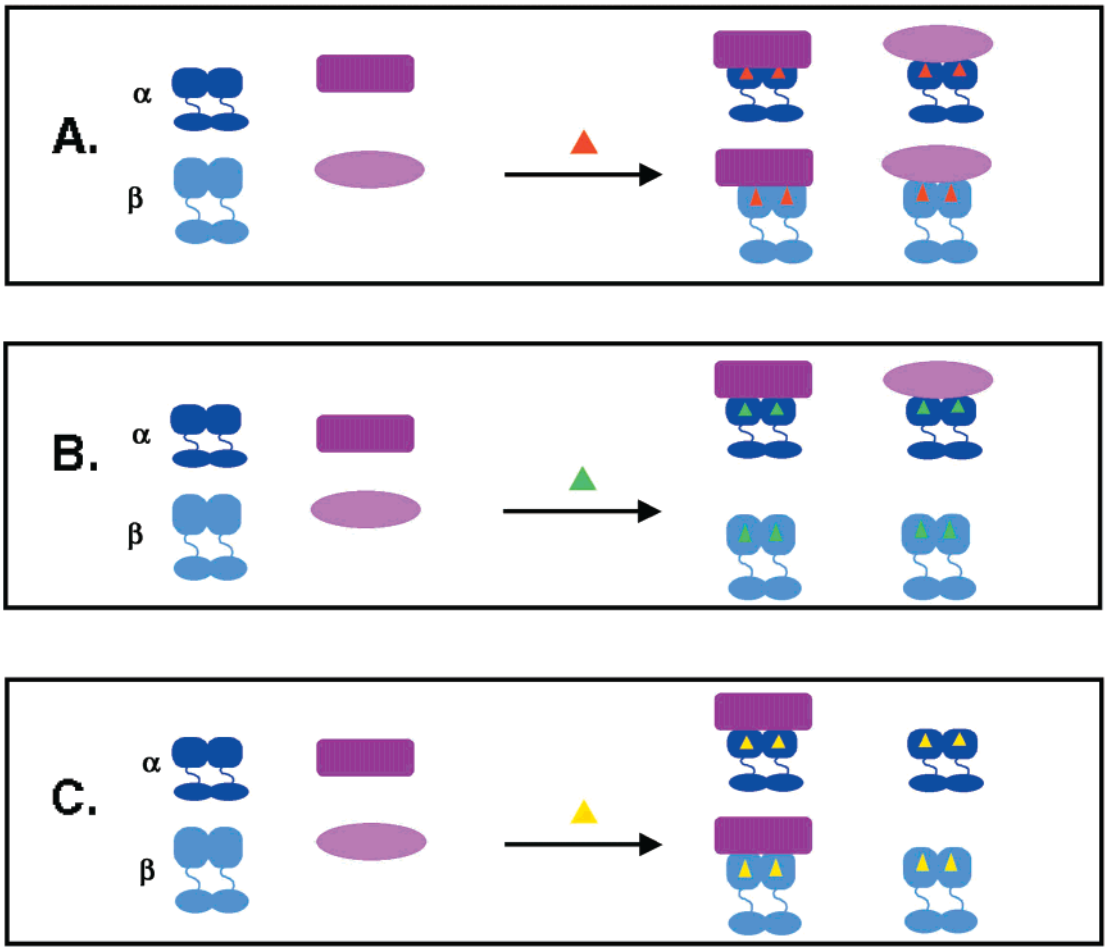

FIGURE 7: Ligands selectively affect nuclear receptor functions. (A) Some ER ligands are not selective in their ability to affect ER interaction with coactivators. They promote, like 17β-estradiol, or inhibit, like 4-hydroxytamoxifen, raloxifene, or ICI-182,780, ER interaction with all the tested coactivators. (B) Some ER receptor ligands are receptor selective and, when bound, have different effects on the activity of ER α and β. WAY164397 enhances ER β interaction with SRC1 and SRC3, but exhibits a minimal effect on interaction of ER α with these coactivators. (C) ER ligands can be coactivator specific. Genistein enhances ER α and β interaction with SRC1 and SRC3, but demonstrates a minimal effect on receptor interaction with DRIP205 and CBP.

fact that separate LXXLL motifs interact with ERs with only micromolar affinity while NRIDs interact with receptors with affinities in the nanomolar range, indicate that LXXLL motifs represent just an anchorage for the binding of the receptors. Once bound, the receptor probably contacts other motifs of the coactivator molecule. We think that these additional interactions contribute significant amounts of energy to receptor−coactivator interaction, increasing its affinity. It is also possible that they contribute to ER−coactivator selectivity. Identification of these motifs represents an important goal for future studies.

We have evaluated the affinities of ER α and β interactions for p160 family members. Our results indicate that both receptors have strong affinity preferences for particular coactivators and that SRC3 is the preferred partner exhibiting the highest-affinity interaction with ER α. ER β interacts with p160 family members with lower affinity, which may explain why its transcriptional activity is lower. These results suggest that ER-mediated transcription is not driven by a random mixture of ER−coactivator complexes.

We have also evaluated the role of ligands in ER−p160 interactions. In accordance with previous studies, we found that 17β-estradiol enhances the affinity of ER α and β for all tested coactivators. 4-Hydroxytamoxifen, raloxifene, and ICI-182,780 inhibit this interaction. Therefore, the nature and concentration of the bound ligand determine the concentration of the receptor−coactivator complex and, as a consequence, the rate of transcription. We have shown that some ligands are not selective in their ability to affect ER interaction with coactivators (Figure 7A). They promote, like 17β-estradiol, or inhibit, like 4-hydroxytamoxifen, raloxifene, or ICI-182,780, ER interaction with all tested coactivators. A good correlation was found between their binding and stimulation or inhibition of ER−coactivator interactions.

Differential expression of ER α and β suggests that receptor specific ligands may possess tissue selective activity. We have characterized a functionally selective ER β ligand. This compound binds to both receptors and enhances interaction of ER β with SRC1 and SRC3 but, most interestingly even at saturating levels, exhibits a minimal effect on interaction of ER α with these coactivators. We conclude that receptor ligands can be functionally selective, and when bound have different effects on receptor activity (Figure 7B).

We have evaluated a number of ER ligands that are known to have tissue selective activity and are able to activate estrogen receptors. One of these compounds, genistein, is a member of the isoflavonoid family and a prominent component in soy products. Isoflavones are weak estrogens but possess other potentially important biological attributes

independent of their ability to bind to the estrogen receptor. Genistein is an inhibitor of several steroid-metabolizing enzymes, tyrosine kinases, and topoisomerases II and I. We have shown that genistein is a coactivator specific ligand. It enhances ER interaction with SRC1 and SRC3, but demonstrates a minimal effect on receptor interaction with DRIP205 and CBP (Figure 7C). These results support the possibility that ligands can be created which selectively affect nuclear receptor interaction with other transcription factors. We believe that these compounds may have an important pharmacological activity.

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