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Ligand-Selective Inhibition of the Interaction of Steroid Receptor Coactivators and Estrogen Receptor Isoforms

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Summary

Ligand-dependent nuclear hormone receptor (NR) signaling requires direct interaction between NR and the steroid receptor coactivators (SRC). Herein we utilize a library of SRC2 peptidomimetics to select for specific inhibitors of the interaction of SRC2 with the two estrogen receptor (ER) isoforms, ERα and ERβ, in the presence of three different ligands: 17β-estradiol, diethylstilbesterol, and genistein. The pattern of inhibitor selectivity for each ER isoform varied depending upon which ligand was present, thus demonstrating that the ligands exert unique allosteric effects upon the surface of the SRC binding pocket. Several of the lead compounds are highly (>100-fold) selective for blocking the binding of SRC2 to ERα, in preference to ERβ, in the presence of one ligand and therefore may prove useful for decoupling ERβ signaling from ERα signaling.

Introduction

The nuclear receptor (NR) superfamily of hormone-induced transcription factors integrates specific signals to properly regulate distinct physiological responses including development and dynamic homeostasis [1, 2]. Misregulation of these signals has been directly correlated with disease states, including various forms of cancer and metabolic diseases [3]. The NR can act as simple transcription factors (where they are the only DNA binding factor in the complex), as complex factors (where they work in concert with other DNA binding factors), or as tethered factors (where their interaction with DNA is mediated by another protein) [4]. In ligand-dependent signaling, they generally function to activate transcription at simple elements by the recruitment of a family of cofactors, the steroid receptor coactivators (SRCs), through the binding of the SRCs' nuclear receptor interaction domain (NID) [3, 5] (Figure 1). The SRCs themselves have several well-characterized activation domains: one that recruits CBP/p300 [6], another that recruits CARM, an arginine methyltransferase [7], and a domain with histone acetyl transferase activity. There are at least three SRCs: SRC1, SRC2/GRIP1/TIF2, and SRC3/ACTR/Rac3/pCIP/AIB1 [8–16]. A unified nomenclature, in which the proteins are referred to as SRC1, SRC2, and SRC3, has been proposed by O'Malley and is adopted in this report. Homozygous disruption of the genes encoding each SRC causes fairly distinct phenotypes in mice, thus suggesting the possibility of distinct mechanisms or activities for each SRC [17, 18]. However, there is evidence of compensatory upregulation of SRC2 following disruption of SRC1, indicating the possibility of partially overlapping function. The underlying biochemical mechanisms that may allow for conservation or separation of SRC function in ligand-dependent signaling are poorly understood. Inhibitors that would allow selective disruption of particular NR•SRC interactions in vivo would be very useful in elucidating such mechanisms.

Genetic and biochemical studies have identified the NID of the SRCs, which contains multiple interaction motifs known as NR boxes, each with a consensus sequence of $L_1XXL_2L_3$ [3, 5]. The NR•SRC protein-protein interaction is highly conserved across the NR superfamily, with these coactivators seemingly shared by most or all of the NR proteins. An analogous motif (I/LxxII) has been identified for corepressors SMRT and NCOR, and the binding sites for coactivator and corepressor may be partially overlapping [19, 20]. It appears that corepressors can also utilize an analogous motif to mediate their interaction with NR in some contexts [21, 22].

Each NR box of the SRCs NID can have different affinities for a particular NR•ligand•promotor triad and can bind in a cooperative [23] or noncooperative [24, 25] manner. Despite recent findings of additional interactions of SRC with NR outside the NID [26], blocking the interaction of NR and SRC in cellular models by overexpression of fusions of SRC NR box peptides inhibits gene transcription from elements normally responsive to hormone-induced signaling [27]. Short NR box peptides and α-helical peptidomimetics containing the NR box sequence can effectively compete with the entire NID of the SRC2 [24]. For example, hERα, hERβ, and hTRβ share the use of SRC2 despite the fact that they regulate entirely different gene transcription pathways [28]. In vitro, hTRβ and both ER isoforms tightly bind the second NR box of SRC2 (SRC2-2), ^{685}EKHKIL_1 ERL_2L_3KDS^{697}, in the presence of their native ligands thyroid hormone (T_3) and estradiol (E_2), respectively. The ER isoforms also interact with SRC2-1 with lesser affinity while the TRs interact with SRC2-3.

The estrogen receptors have distinct biological responses to different natural and synthetic estrogen-like ligands [29–31], so-called selective estrogen response modulators (SERMs). The effect of the ligand depends primarily on the ability to alter the structure, stability, and interactions of the receptor LBD [32]. Estrogen and estrogen-like ligands function by binding with high affinity to the ligand binding domain (LBD) and partially filling the hydrophobic core of the domain. The ligands allosterically define the overall LBD structure by creating a conformational change in the LBD that promotes or inhibits the binding of coregulator proteins. The ER isoforms bind SRC2-2 when liganded with either the syn-

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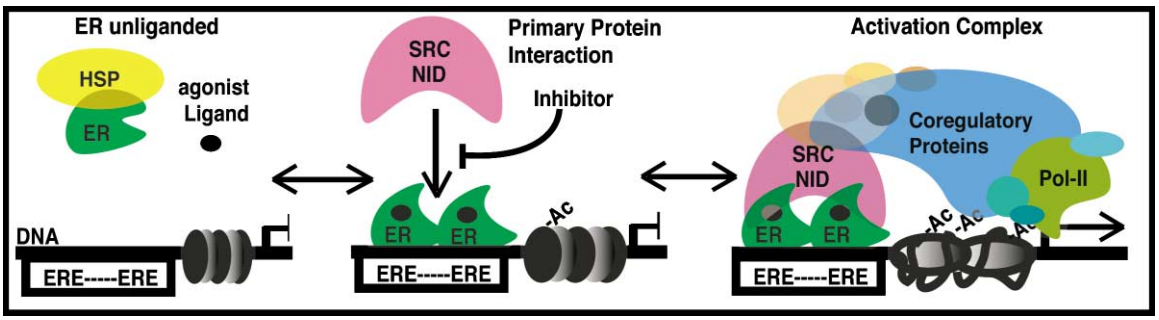

Figure 1. Ligand-Dependent Nuclear Receptor Regulatory Transcription Complex Assembly

Schematic model of the dynamic assembly of the transcription activation complex by agonist-bound estrogen receptor (ER) and function of steroid receptor coactivator (SRC) binding inhibitors. From left to right: In the absence of ligand, chromatin is unmodified and transcription at the DNA estrogen response element (ERE) is unaltered. Binding of agonist ligand to the ligand binding domain (LBD) of ER induces a conformational change in ER, leading to translocation and homodimerization on the ERE. Liganded ER on the ERE recruits SRCs using the NR box ($L_1XXL_2L_3$) of the nuclear receptor-interacting domain (NID). Subsequently, the ER•SRC complex recruits other coregulator proteins and transcription factors to form the activation complex where chromatin is modified and transcription of the ERE gene commences. Direct competitive inhibition of SRC binding to NR will block the initial step of activation complex formation and thus prevent transcription.

thetic partial agonist diethylstilbesterol (DES) or with the phytoestrogen genistein (Gen) [33]. Depending on the physiological context, the receptor isoform, and presence of particular coactivators, this combination may result in an estrogenic, partial estrogenic, or an antiestrogenic response [34]. Until now, the focus of studies of ligand allosteric effects has been on gross structural changes of the LBD, such as the repositioning of helix 12 and its ability to create or conceal the SRC binding pocket.

Structurally, the interactions of hERα and hTRβ with the SRC2-2 peptide are very similar to those of other NR•coactivator structures solved to date [22, 24, 32, 35–37]. Each seems to interact through similar surfaces with a shallow hydrophobic groove on the NR surface binding to an induced fit, amphipathic, α-helical motif on the SRC2-2 NR box, burying the conserved three leucines on the hydrophobic face of the NR box helix. While each of the leucines in the NR box is critical to the interaction [38], in vivo specificity in recruiting a particular SRC appears to be induced by the sequences immediately flanking the NR boxes [27, 33, 37, 39] rather than the geometry of the leucine side chains. Manipulation of NR box peptide sequence outside of the conserved $L_1XXL_2L_3$ motif has afforded selective peptide inhibitors of the interaction of particular NR and SRCs, presumably by taking advantage of these extended interactions [27, 40, 41]. However, such extended elements would not be available to a small molecule disrupting the interaction, and it is therefore uncertain how such findings reflect upon the development of such a drug.

As our approach targets such drugs, it was paramount to identify subsite-specific differences within the small hydrophobic pocket that binds the NR box that could be effectively utilized by a small molecule. We therefore rationally designed a series of SRC2-2 NR box mimetics [42] with one of three leucines substituted on a constrained peptidomimetic scaffold [43]. Despite the high degree of structural conservation in these interfaces, it is apparent from our recent studies that there are subtle differences in the hydrophobic interface of each NR. These unique characteristics of each receptor interface allow nonnatural LXXLL peptidomimetics to selectively inhibit the interaction. For example, when targeting three homologous nuclear receptors (hTRβ, hERα, and hERβ) liganded with their physiological ligands, we found ten peptidomimetics that were selective for hERα, one that was selective for hERβ, and one that was selective for hTRβ by at least 10-fold [42].

Herein we report our analysis of the structural foundation of this observed selectivity and the examination of the ligand-dependent allosteric modulation of the ER: SRC2-2 interface by three related ligands: estrogen, diethylstilbesterol, and genistein. In vitro competition studies show that these binding sites are allosterically modulated by the different ligands in a way that permits the differential selection of SRC2-2 peptidomimetic inhibitors without significantly altering the binding of the native SRC2-2 NR box.

Results and Discussion

Structural Analysis of NR•SRC Interactions

At the protein fold level, the differences between the NR coactivator interfaces (Figures 2B–2D) of T_3•hTRβ•SRC2-2 [24], DES•hERα•SRC2-2 [32], and E_2•hERα•SRC2-3 [44] appear minimal. Each of the SRC2 peptide main chain rmsd's is less than 1.0 Å, and the receptor main chain rmsd's are less than 2.0 Å. However, upon closer investigation, we find that there are differences in the side chain positions within each pocket and within pocket surfaces that alter the electrostatics, topography, and the location of solvent-accessible volume between the pockets of each receptor (Figures 2E and 2F). In particular, comparing hTRβ and hERα reveals that hERα (Figure 2C) has a ridge with a continuous electrostatic potential adjacent to the L_2 subpocket, whereas hTRβ (Figure 2B) has a subpocket that is sterically hindered by a more significant plateau with a gradient of electrostatic potential. Additionally, a pronounced ridge in hTRβ creates more steric hindrance between the L_1 and L_3 subpockets than what is present in hERα. A total of 19 amino acid side chains on the surface of both

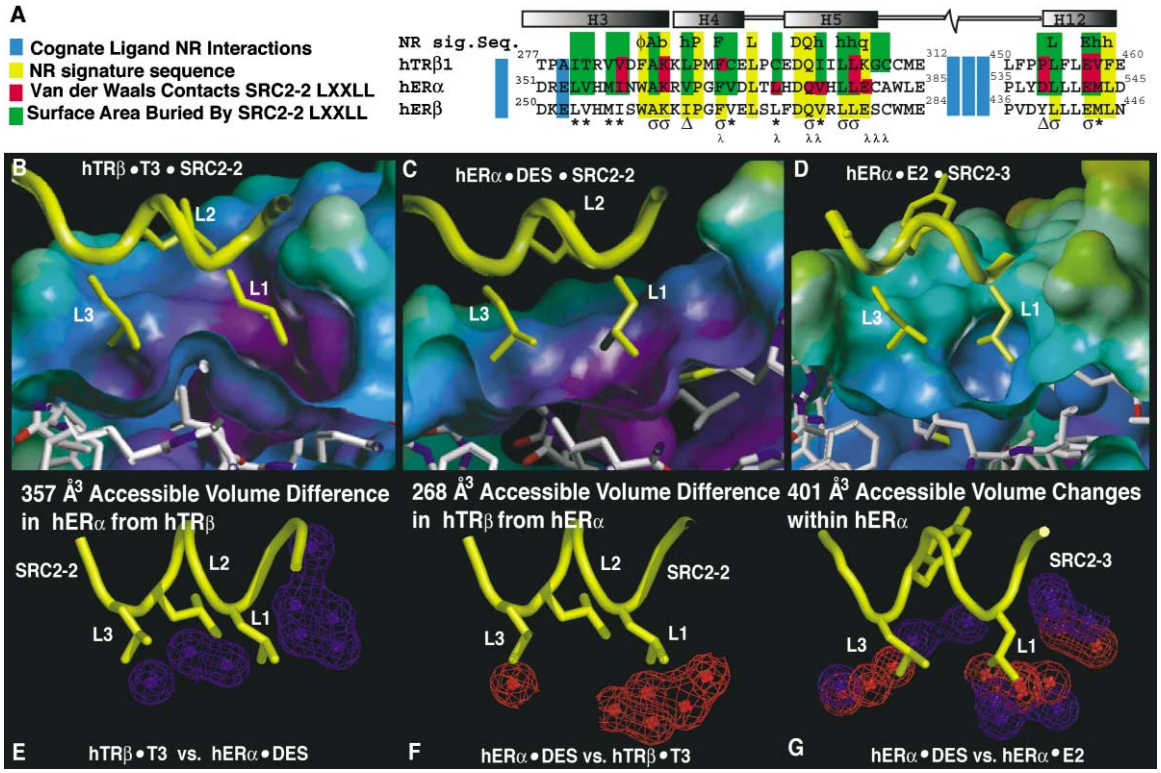

Figure 2. Phylogenetic and Structural Comparison of Three Cocrystal Structures Highlighting Significant Differences in the Subsites of Each Nuclear Receptor Interface

(A) Alignment of the primary amino acid sequences that make up the coactivator binding pockets of the receptors hTRβ, hERα, and hERβ. The secondary structure of the proteins is identified in gray with each helix numbered according to the convention for NR. Amino acids are shown in single-letter format with residue numbers from the receptor primers beginning and ending each segment. The NR signature sequence is shown on top with (ϕ) indicating an aromatic residue, (A) indicating alanine, (b) indicating a basic residue, (h) indicating any hydrophobic residue, (P) indicating proline, (L) indicating leucine, (Q) indicating glutamine, and (q) indicating a charged residue. Blue denotes areas of the receptor that make contact with the respective native ligands of each receptor. Red denotes residues identified to form VDW interactions with the leucine side chains of SRC2-2 in the respective crystal structures. Green denotes residues that compose the hydrophobic pocket and are buried in the crystal structures. An asterisk indicates residues that are buried in the respective complexes and are different between the hTRβ and the two ER isoforms but are the same between the isoforms; (σ) indicates the residues that are the same across all the receptors; and (Δ) indicates residues that different across each receptor. This clearly demonstrates differences in the side chain character of the SRC binding pocket. (λ) indicates residues that have a different type of interaction with the coactivator.

(B–G) Cocrystal structure of the SRC NR box binding pocket in: (B) T_3•hTRβ•SRC2-2[1], (C) DES•hERα•SRC2-2[2], and (D) E_2•hERα•SRC2-3. The SRC binding pocket in each cocrystal structure is shown as a cross-section through the L_1 and L_3 recognition pockets to highlight structural and electrostatic differences within the highly conserved binding pockets. The three leucine side chains of the SRC2-2 ($L_1XXL_2L_3$) and the SRC2-3 (LLTXXLL) motifs (yellow wire frame) are labeled L_1 L_2 L_3. Each receptor Conolly surface was generated with a probe of 1.4 Å radius and color coded to induce a continuous electrostatic gradient (*Sybyl*). These views demonstrate clear significant differences in steric structure and electrostatics between the hTRβ and hERα SRC binding pockets (B versus C) as well as potential plasticity within hERα surface (C versus D). These structural and volumetric differences between each comparison are highlighted in (E) and (F) with difference maps consisting of 2.2 Å radius spheres that differentially fill each pocket of each receptor. (E)–(G) are oriented the same as (B)–(D): (*PyMOL*) spheres of 2.2 Å radius filled the solvent accessible volume of each receptor pocket. Spheres were compared and removed if shared between receptor pockets, leaving only those that were present in one structure and not the other. In (E), blue web spheres highlight the location of eight spheres totaling 357 Å^3 of volume that is available in the hERα pocket and not in the hTRβ pocket. In F, six red spheres locating 268 Å^3 of volume that is available in hTRβ and not hERα. (G) shows a similar comparison between the pockets of (C) DES•hERα•SRC2-2 and (D) E_2•hERα•SRC2-3, with blue denoting the location of nine spheres totaling 401 Å^3 present in the pocket of DES•hERα•SRC2-2 and not in E_2•hERα•SRC2-3; and the reverse comparison also showing nine red spheres but in different locations. This highlights that the pocket has changed in topography without a change in accessible volume, possibly due to the change in ligand or in response to the different SRC-interaction motif.

hERα and hTRβ are either buried by SRC2-2 or form direct contacts with the NR box (Figure 2A). Only eight of these amino acid residues are conserved between the two receptors. Fifteen of the 19 residues are different between TRβ and ERα by sequence or degree of physical interaction with the SRC2-2 NR box leucines (L354I, V355T, M357V, I358V, V364L, V368C, L372C, V376I, E380K, D538P, M543V F367F, and Q375Q; ERα numbering, see Figure 2). Eight of these form unique direct contacts (underlined) with the leucines of SRC2-2 in one receptor and not the other, while two residues (C381G and A382C) are buried in the TR pocket but not with ERα. Four nonconserved residues (L354I, M357V, I358V, and V364L) are conservative substitutions, while six (V355T, V368C, L372C, C381G, A382C, and D538P) exhibit large changes in shape, electrostatics, or hydrogen

bonding potential. The presence of four surface-exposed cysteines in the coactivator binding pocket on TR is somewhat surprising and may point to a potential for regulation of coactivator binding by oxidative post-translational modifications such as nitrosylation, which have been observed with other transcription factors [45]. The relative location and substitutions of proline and glycine between ER and TR are of some interest and may reflect potential for greater flexibility of secondary structure in ER.

The two ER isoforms have 59% homology in the ligand binding domain [46], suggesting that achieving selectivity between them would be difficult. Assuming that ERα and ERβ interact with the SRC2-2 peptide similarly (no cocrystal structure of ERβ•SRC2-2 is available), only 2 of the 19 residues lining the pocket are different between the ER isoforms. V364I is a fairly conservative change, while D538Y (hERα numbering) is a major change. Two spatially adjacent substitutions (C381S and A382C) present changes in electrostatic or hydrogen bonding potential.

To aid analysis, we generated volume maps of the NR box binding sites for the receptors and quantitatively subtracted them from one another to compare differences (Figures 2E–2G). This analysis revealed that there is 357 Å^3 available in the ERα pocket that was not addressable from the hTRβ pocket (Figure 2E) and 268 Å^3 available in hTRβ but not in hERα (Figure 2F). A similar comparison of hERα with two different ligands and different SRC2 peptides, DES•hERα•SRC2-2 and E_2•hERα•SRC2-3 [44], shows significant differences within the pocket (Figure 2G). There is not a change in the overall volume of the pocket but rather a change in the location of solvent accessible volume. However, this last comparison is complicated by the multiple differences in the crystallization conditions of hERα (including ligand and peptide partner), which prevent unambiguous assignment of surface differences to the change in the ligand, the change in NR box peptide sequence, or both.

Overall, this analysis confirmed that, while the differences in the coactivator binding pockets are subtle, there is typically at least 250 Å^3 in volume change and accompanying changes in electrostatics from NR•ligand pair to pair. In general, the large number of significant changes in residues involved in coactivator binding partially explains our ability to find specific inhibitors targeted at this pocket [42]. However, as will be discussed below, they do not allow for computational predetermination of selectivity.

Screening for Ligand•NR-Selective Peptidomimetics

Previous reports of the effects on ERα and ERβ interactions with SRC2-2 peptides in the presence of these ligands indicated that there was little change in the recruitment of the individual boxes [33]. While the ligands do not significantly perturb the binding of the native LXXLL NR box motifs, our analysis indicated that the hydrophobic pocket was being modulated in such a way that the binding of other nonnatural leucine mimetics would be significantly effected. To explore this idea, we screened the library against hERα and hERβ in the presence of different ligands, including a synthetic estrogen analog diethylstilbesterol (DES) and the phytoestrogen genistein (Gen), both partial agonists that have been shown to recruit SRC2-2 to both ER isoforms.

This peptidomimetic library utilizes an α-helical scaffold (Figure 3A) [43]: a conformationally constrained NR box peptide **1{37,37,37}** that functionally disrupts the interaction of SRC2 and hTRβ. Peptidomimetic **1{37,37,37}** binds to hTRβ 15-fold better than the native SRC2-2 peptide: 10.8 μM and 0.79 μM, respectively [43]. We utilized this lead compound as a constrained helical scaffold for the presentation of a library of compounds with nonnatural amino acid side chains that mimic each of the three leucines within the LXXLL motif. Each member of this library was comprised of a unique compound, synthesized in parallel, that had one of the leucines changed to a nonnatural amino acid—predominantly substituted phenylalanines and phenylglycines. Each library member was purified by RP-HPLC, and purity and identity were confirmed prior to use [42].

The competitive ability of each of the library members to inhibit the binding of SRC2-2 to the two ER isoforms was assessed using a fluorescence polarization equilibrium competition assay as previously described [42]. The OG-SRC2-2 probe bound to each receptor in a ligand-dependent manner with Kds of 299 nM, 310 nM, and 450 nM for E_2•hERα, DES•hERα, and Gen•hERα, and 370 nM, 450 nM, and 520 nM, for E_2•hERβ, DES•hERβ, and Gen•hERβ, respectively. The lead compound **1{37,37,37}** competed for each of these interactions with SRC2-2 with IC_{50} values of 120 nM, 290 nM, and 110 nM for E_2•hERα, DES•hERα, and Gen•hERα, and 151 nM, 261 nM, and 350 nM for E_2•hERβ, DES•hERβ, and Gen•hERβ, respectively. There is little selectivity between isoforms with the lead compound relative to the natural affinities of SRC2-2. The entire library was then screened with each receptor•ligand pair. Surprisingly, many of the compounds that were previously identified to inhibit the E_2•hERα interaction with SRC2-2 did not inhibit interaction with either one or both of the DES- or Gen-bound hERα. Likewise, several of the compounds that did not seem to target E_2•hERα inhibited the SRC2-2 interaction with DES•hERα or Gen•hERα (Figure 3B).

The selectivity of twelve inhibitors changed by 10-fold for hERα in a ligand-dependent fashion. At L_1, the *para*-fluorophenylglycine **1{3,37,37}** effectively targeted only the DES•hERα form of the receptor. At L_2, the *meta*-trifluoromethylphenylglycine **1{37,9,37}** targeted E_2•hERα, the cyclohexylalanine **1{37,20,37}** was selective for both the E_2 and DES liganded forms of hERα, the cyclopentylphenylglycine **1{37,28,37}** was selective for DES over Gen but not between DES and E_2, and tryptophan **1{37,34,37}** is highly selective for the DES-bound form of hERα. At the L_3 position, three phenylglycine compounds were selective for E_2: 2,3-Difluorophenylglycine, 3,4-Difluorophenylglycine, and 1,5-Difluorophenylglycine, **1{37,37,4}**, **1{37,37,5}**, and **1{37,37,6}**. The *ortho*-chlorophenylalanine **1{37,37,14}** and tryptophan **1{37,37,34}** selected for the DES•ERα ligand (Figure 3C).

The specificity of sixteen inhibitors changed for hERβ between the three ligands. At the first leucine position L_1, the phenylglycine **1{1,37,37}** was selective for DES and Gen over E_2. The cyclohexylalanine **1{20,37,37}** and cyclopentylphenylalanine **1{29,37,37}** were selective for both E_2 and DES over Gen. At position L_2, phenyl glycine

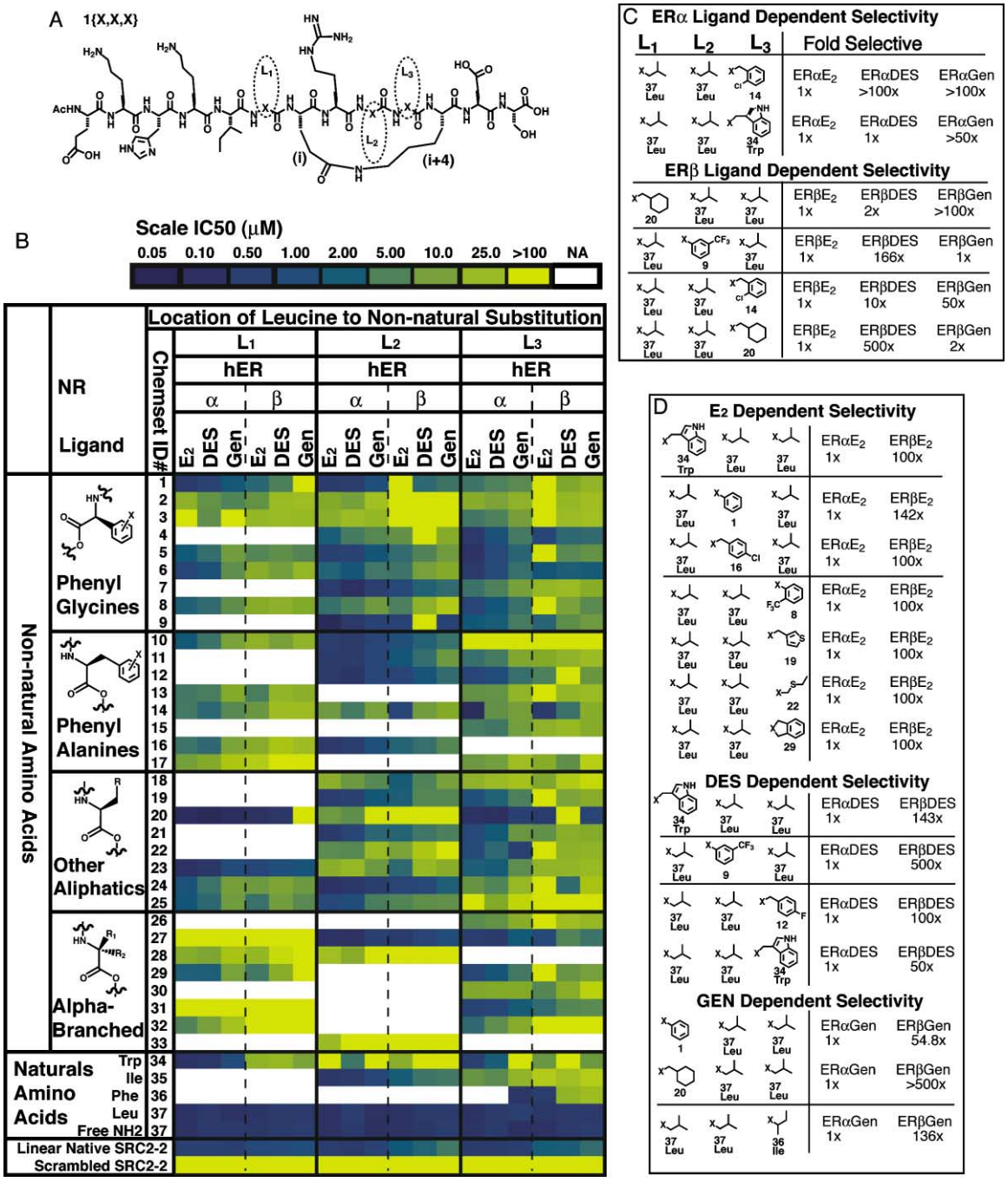

Figure 3. The Scaffold Design and Estrogen Receptor Isoform Ligand-Dependent Inhibitory Selectivity Profile of the SRC2-2 Proteomimetics

(A) The structure of the proteomimetic library scaffold **1{X,X,X}** with diversity positions indicated by L_1, L_2, and L_3. 1{37,37,37}: L1 = L2 = L3 = LEU.

(B) The relative equilibrium 50% inhibitory concentration (IC_{50}) for each library member for competition of the SRC2-2 peptide from each ER isoform (hERα or hERβ) in the presence of three ligands (E_2, DES, Gen) as determined by two separate quadruplicate in vitro fluorescence polarization evaluations is presented in a colorimetric scale that has been normalized to the IC_{50} values of the lead compounds **1{37,37,37}**, with gold indicating an IC_{50} >100 μM, dark blue indicating an IC_{50} of <100 nM, and gradations of color between the two indicating intermediate IC_{50} values. White boxes denote compounds whose synthesis was not achieved. Individual nonnatural amino acids are arrayed on the y axis and numbered according to each nonnatural side chain, as previously described. The x axis depicts the position of nonnatural amino acid substitution (L_1, L_2, or L_3) and the NR tested. Actual values and the 95% confidence range are presented in Supplemental Tables S1–S3.

(C) Selective inhibitors with >50-fold selectivity. The selectivity profile of SRC2-2 proteomimetic inhibitors that are specific to one NR/ligand by >50-fold due to individual side chain substitutions at one Leu position. Selectivity among the tested NR is indicated on the right side with the fold decrease in the IC_{50} relative to the IC_{50} against the receptor for which the compound is most selective. Structures of six library members that change their selectivity for hERα and hERβ in a ligand-dependent fashion (hERα and hERβ were tested in the presence of estrogen (E_2), the SERM diethylstilbesterol (DES), and the phytoestrogen genistien (Gen).

(D) The structures of 14 inhibitors that were selective for hERα over hERβ with each ligand.

1{37,1,37} was selective for DES and Gen over E_2, while **1{37,4,37}** and **1{37,5,37}** were selective for both E_2 and Gen over DES and **1{37,14,37}** was selective for E_2. At position L_3, five phenyl glycines were selective for both DES and Gen over E_2: **1{37,37,1}, 1{37,37,2}, 1{37,37,3}, 1{37,37,5}**, and **1{37,37,8}**; of the phenylglycines, **1{37, 37,12}** was selective for E_2 and Gen, while **1{37,37,14}** was selective for E_2; **1{37,37,20}** was selective for E_2 and Gen over DES; **1{37,37,24}** was selective for DES; and **1{37,37,29}** was selective for DES and Gen over E_2. Six compounds changed their affinity for hERβ or hERα in a ligand-selective manner by >50 fold (Figure 3C).

A total of 19 inhibitors were selective for hERα over hERβ by 10-fold or more when the receptors were liganded with E_2: four with substitutions at L_1 (**1{6,37,37}, 1{10,37,37}, 1{32,37,37}, 1{34,37,37}**), four at L_2 (**1{37, 1,37}, 1{37,9,37}, 1{37,16,37}, 1{37,20,37}**), and 11 at L_3 (**1{37,37,1}, 1{37,37,3}, 1{37,37,8}, 1{37,37,13}, 1{37,37, 19}, 1{37,37,22}, 1{37,37,24}, 1{37,37,26}, 1{37,37,29}, 1{37,37,32}, 1{37,37,34}**). Fifteen inhibitors were selective for hERα over hERβ when bound to DES: three with substitutions at L_1 (**1{16,37,37}, 1{32,37,37}, 1{34,37,37}**), seven at L_2 (**1{37,4,37}, 1{37,7,37}, 1{37,9,37}, 1{37, 19,37}, 1{37,20,37}, 1{37,34,37}, 1{37,35,37}**), and five at L_3 (**1{37,37,12}, 1{37,37,23}, 1{37,37,27}, 1{37,37,32}, 1{37,37,34}**). Six were selective for hERα in the presence of Gen: three with substitutions at L_1 (**1{1,37,37}, 1{20, 37,37}, 1{34,37,37}**), one at L_2 (**1{37,11,37}**), and two at L_3 (**1{37,37,22), 1{37,37,36}**). Two of the inhibitors were selective for hERβ: the previously identified *ortho*-chlorophenylalanine at L_2, **1{37,14,37}**, in the presence of E_2, and tryptophan at L_3, **1{37,37,34}**, in the presence of Gen. Fourteen of these inhibitors were specific to hERα with >50-fold selectivity over hERβ in the presence of one of the three ligands (Figure 3D). This set represent the most promising leads for physiologic study or novel therapy. Each of these compounds has the potential to highly selectively abrogate ERα signaling induced by a particular ligand without affecting that of ERβ.

Analysis of Structural Determinants of Selectivity

The interaction modes of a number of the selective inhibitors were evaluated using DOCK (Figures 4A–4F). Comparing the results of the in silico CombiDOCK screening and the in vitro competition data of the same library reveals that while this method was very useful for enhancing the likelihood of the library producing competent inhibitors, it was not effective for predicting selectivity between the NR. Many of the compounds scored similarly between the receptors and in almost identical minimized positions. The only correlation related to selectivity that is apparent between the in silico and in vitro binding studies is the size of the side chains. Larger residues at positions L_1 and L_3 score slightly better when DOCKed with DES:hERα than T_3:hTRβ, and that trend is apparent in the competition assays. Several DOCKed inhibitor structures seemed to take advantage of the differences that were identified by the structural and volumetric analysis (Figures 2B–2G). Peptidomimetics **1{37,8,37}** and **1{37,37,14}**, for example, bury solvent accessible volume that was identified in ERα and not in TRβ (Figure 4). However, a number of the selective inhibitors would not fit into the pocket unless the van der Waals constraints were relaxed by 0.2 Å (data not shown). These compounds represent selectivity that could not be predicted computationally.

ERα clearly tolerates larger groups within the NR box binding pocket than the crystal structures indicate would be possible. This finding is most likely explained by one of two models: (1) the SRC peptidomimetic inhibitors are binding in a different orientation than what is seen from the native peptide crystal structures, and/or (2) the surface of hERα is plastic and is responding to the inhibitors in a way that permits these compounds to unveil new subsites that are energetically favorable. The latter may be possible due to the reduced entropic cost of peptide helix formation translating into additional binding energy that perturbs the receptor surface differently than the crystal refined state.

Discussion

The SRC binding pockets of NR have evolved to bind to a simple hydrophobic $L_1XXL_2L_3$ consensus motif while relying upon differences in SRC sequences flanking the NR box to convey selectivity in vivo. Our studies show that ER has a tolerance and even strong selection for aromatic moieties replacing the leucine side chains when presented on an appropriate peptidomimetic scaffold. This observation is in stark contrast to prior studies with expressed peptide libraries [27, 41] that did not select for phenylalanine, possibly due to its negative effect on the helicity of unconstrained peptides. Our studies reveal that the NR box binding pockets of the NR contain significant differences in shape and electrostatics that allow competitive inhibitors that mimic the NR box to act selectively between NR and even between the same NR when a different is ligand bound.

These results were only obtained by utilizing a constrained scaffold that removes the entropic cost of helix formation in this induced-fit α helix protein-protein interaction. Other methodologies utilizing linear peptide libraries composed of natural amino acids have been unable to select for side chains other than leucine with these receptors [27, 41]. Linear peptides with a phenylalanine substituted at each of the leucine positions of a similar peptide from the SRC2-2 (KHKI**F**HRLLQDSS, KHKILHR**F**LQDSS, and KHKILHRL**F**QDSS) [24] reduced the competitive ability of the SRC2-2 peptide by 60- to 100-fold, the worst being the L_1 substitution [24]. Here, we clearly show that such phenylalanine replacements, as well as substituted phenylalanine analogs, are able to inhibit SRC2-2 binding quite well. Tryptophan, which has not been selected in genetic screens, also clearly works well in a constrained peptide background. It is our belief that these larger side chains fit quite well into the interface, but only when presented on a properly folded α helix. This apparent disparity suggests that the previous hypothesis that these NR cannot tolerate the larger side chains may be incorrect. An alternate explanation consistent with both sets of data is that the effect of each residue on the helicity of the given peptide sequence, in combination with the size-shape complementarity to the receptor surface, controls affinity. This hypothesis begs the question: are their other, yet to be

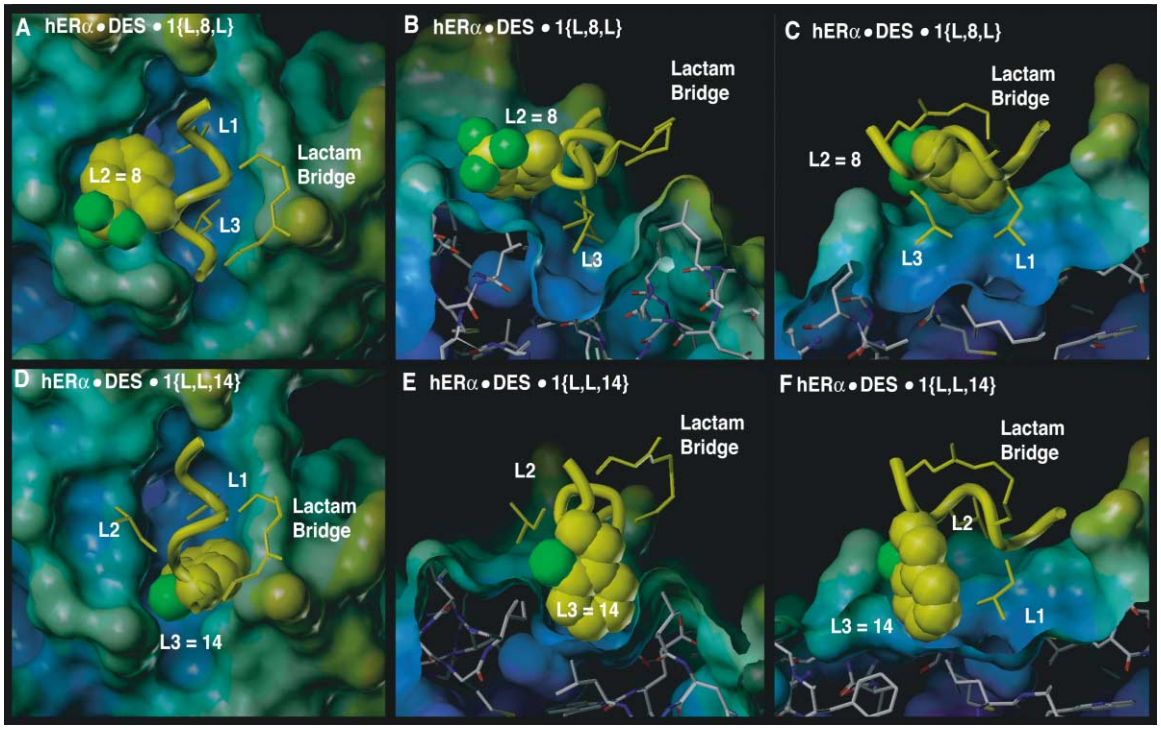

Figure 4. DOCKed Structures of Two Selective Inhibitors

(A–C) The energy minimized structure of the hERα•DES-selective inhibitor **1{37,8,37}** with *o*- trifluoromethylphenylglycine replacing the second leucine of the NR box. **1{37,8,37}** scored the highest in the docking studies with both receptors hERα•DES and hTRβ•T3.

(D–F) The inhibitor **1{37,37,14}** with *o*-chlorophenylalanine replacing the third leucine of the NR box, which scored better with hERα•DES than with hTRβ•T3. Each of the DOCKed inhibitor structures was similar in orientation to that of the crystallographic coordinates of SRC2-2. The rmsd values were as follows: **1{37,8,37}** at $<$0.2 Å and **1{37,37,14}** at $<$0.6 Å. A number of selective inhibitors would not DOCK in the normal orientation without relaxation of the restrictions to maintain the normal relationship between the peptide backbone and the receptor.

identified, NR interactions at this site with proteins that have different sequences? These could easily include larger side chains that are stabilized into α-helical secondary structures by their tertiary structures. A second interesting question arises: how do the flanking sequences around the NR box LXXLL motif drive selectivity? Is it due to their direct contacts with the receptor surface, as has been previously suggested [27, 41], or is it due to some effect on helicity? The notion that they make particular contacts with the receptor surface to drive the interactions has been difficult to verify both structurally and biochemically. The flanking sequences could engender different levels of helicity by effecting helix dipole or through specific contacts. Additionally, there may be transient interactions with the surface of the receptors that seed the induction of helicity, as has been recently suggested [44].

Another finding arising from our data is that the partial ER agonists are allosterically modulating "subsites" of the leucine recognition pockets without perturbing the interactions of the native LXXLL NR box sequences. This suggests that one could potentially simultaneously target one receptor with, for example, an ER isoform-specific ligand and a SRC peptidomimetic inhibitor that is selective for that combination.

A similar failure to evolve tight shape complementarity between receptor and peptide ligand has been observed in the interactions of SH3 domains [47]. It seems like this may be a common situation in signaling interactions that function dynamically with relatively weak affinity for their partners. These systems may represent a particularly tractable subset of protein interactions for inhibitor development. The successful identification of specific inhibitors of the E_2•hERα•SRC2-2 protein interaction utilizing nonnatural α-amino acids in an α-helical peptidomimetic library demonstrates the feasibility of specifically targeting the competition of protein interactions where consensus motifs consist entirely of a small hydrophobic patch. This preliminary study has already yielded inhibitors of ERα interactions with SRC2 that are functionally selective for that isoform in preference to effecting interactions of ERβ.

Significance

Ligand-dependent nuclear hormone receptor (NR) signaling requires direct interaction between NR and the steroid receptor coactivators (SRC), effected by a series of conserved SRC motifs that are composed of three leucines (NR box, $L_1XXL_2L_3$). We have previously shown that peptidomimetics of the second NR box of SRC2 (SRC2-2) can exploit structural differences between the NR box binding pockets of the thyroid hormone receptor β (TRβ) bound to thyroid hormone (T_3), and the two estrogen receptor (ER) isoforms (ERα and ERβ), each bound to estrogen (E2). In this report,

we demonstrate that the same library of SRC2-2 peptidomimetic inhibitors can take advantage of differences between the NR box binding pockets of ERα and ERβ in the presence of three different ligands, 17β-estradiol (E_2), diethylstilbesterol (DES), and genistein (Gen), to afford specific inhibitors. The selection profile for each ER isoform, and between isoforms, changed depending on which ligand was present. Therefore, the simple hydrophobic SRC binding pocket surface on NR is allosterically modulated differently by each ligand. Fourteen of these inhibitors were specific for preventing recruitment of SRC2 by hERα with >50-fold selectivity relative to inhibiting the same interaction for hERβ in the presence of one of the three ligands. Each of these compounds has the potential to decouple ERα signaling induced by a particular ligand from that of ERβ. Because this selectivity is induced solely by manipulating the side chain of one leucine in the analogs, small molecules targeted to this pocket could achieve selectivity for a particular ER•ligand pair by the same mode.

Experimental Procedures

Volumetric Analysis

Publicly available structures T_3•hTRβ•SRC2-2 (Protein database [PDB] ID code 1BSX), E_2•hERα•SRC2-3 (PDB ID code 1GWR), and DES•hERα•SRC2-2 (PDB ID code 3ERD) were utilized for computational evaluation. A Perl script was written to identify solvent accessible volume on the surfaces of protein (A. Shelat, UCSF). The PDB structures were aligned by the Cα and Cβ positions of the three leucines (or two leucines and a tyrosine for 1GWR) with rmsd <0.01 Å of the SRC2 coactivator peptide. The coactivator peptide was removed from the structure and a box of $4 \times 4 \times 4$ Å^3 in dimension was defined with a center defined in PDB coordinate space at the center of the binding pocket. The pockets of both receptors were filled with spheres. Those that came in contact with the receptor surface of a given receptor were removed. The sphere sets were compared between receptors, and those that were identical in both receptors were deleted, while those that were different between receptors were defined as a receptor specific difference. The maps were visualized by imposing a continuous surface onto the remaining spheres.

Peptidomimetic Library Synthesis

The design, synthesis, and characterization of this library was executed as reported [42, 43]. Briefly, orthogonally protected peptidomimetics for the chemset were synthesized on solid support in Robbins Blocks (48 wells/block) with 100 mg resin/well with a resin loading of 0.48 mmol/g as previously described. The conformational constraint was introduced while attached to the resin. After cleavage from the resin and concomitant side chain deprotection, all compounds were purified using HPLC. Compound identity was confirmed by UV spectroscopy, MALDI-TOF MS, and ESI Quadrapole MS.

Fluorescence Polarization Competition

Fluorescence polarization competition was performed as previously reported [42, 43]. Using a LJL Biosystems Analyst (Foster City, CA), experiments were carried out with the following conditions: SRC2-2 probe concentration constant at 10 nM; hERα LBD concentration constant at 200 nM or hERβ LBD constant at 400nM; and ligand concentration constant at 10 μM. The effective binding buffer was 20 mM Tris (pH 7.4), 100 mM NaCl, 40mM KCl, 1 mM DTT, 1 mM EDTA, 0.01% NP-40.

H_6-hERα and H_6-hERβ Protein Expression and Purification

H_6-hERα LBD and H_6-hERβ LBD were expressed in *Escherichia coli* BL21(DE3) as previously described [42].

Supplemental Data

Supplemental data including full experimental details of library composition and quality control, biochemical procedures, and tables of IC_{50} data and CD data is available at http://www.chembiol.com/cgi/content/full/11/2/273/DC1.

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References

1. Baker, M.E. (2003). Evolution of adrenal and sex steroid action in vertebrates: a ligand-based mechanism for complexity. Bioessays *25*, 396–400.
2. Francis, G.A., Fayard, E., Picard, F., and Auwerx, J. (2003). Nuclear receptors and the control of metabolism. Annu. Rev. Physiol. *65*, 261–311.
3. Tsai, M.J., and O'Malley, B.W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. *63*, 451–486.
4. Yamamoto, K.R., Darimont, B.D., Wagner, R.L., and Iniguez-Lluhi, J.A. (1998). Building transcriptional regulatory complexes: signals and surfaces. Cold Spring Harb. Symp. Quant. Biol. *63*, 587–598.
5. Aranda, A., and Pascual, A. (2001). Nuclear hormone receptors and gene expression. Physiol. Rev. *81*, 1269–1304.
6. Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996). p300 is a component of an estrogen receptor coactivator complex. Proc. Natl. Acad. Sci. USA *93*, 11540–11545.
7. Ma, H., Baumann, C.T., Li, H., Strahl, B.D., Rice, R., Jelinek, M.A., Aswad, D.W., Allis, C.D., Hager, G.L., and Stallcup, M.R. (2001). Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. Curr. Biol. *11*, 1981–1985.
8. Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science *270*, 1354–1357.
9. 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). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science *277*, 965–968.
10. Hong, H., Kohli, K., Garabedian, M.J., and Stallcup, M.R. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol. Cell. Biol. *17*, 2735–2744.
11. Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., and Rosenfeld, M.G. (1997). The transcriptional coactivator p/CIP binds CBP and mediates nuclear-receptor function. Nature *387*, 677–684.
12. Blanco, J.C., Minucci, S., Lu, J., Yang, X.J., Walker, K.K., Chen, H., Evans, R.M., Nakatani, Y., and Ozato, K. (1998). The histone acetylase PCAF is a nuclear receptor coactivator. Genes Dev. *12*, 1638–1651.
13. Zhu, Y., Qi, C., Calandra, C., Rao, M.S., and Reddy, J.K. (1996). Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma. Gene Expr. *6*, 185–195.
14. Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O'Malley, B.W. (2000). The steroid receptor coactivator SRC-3

(p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc. Natl. Acad. Sci. USA *97*, 6379–6384.
15. Hong, H., Kohli, K., Trivedi, A., Johnson, D.L., and Stallcup, M.R. (1996). GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc. Natl. Acad. Sci. USA *93*, 4948–4952.
16. Voegel, J.J., Heine, M.J., Zechel, C., Chambon, P., and Gronemeyer, H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J. *15*, 3667–3675.
17. Gehin, M., Mark, M., Dennefeld, C., Dierich, A., Gronemeyer, H., and Chambon, P. (2002). The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. Mol. Cell. Biol. *22*, 5923–5937.
18. Xu, J., Qiu, Y., DeMayo, F.J., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science *279*, 1922–1925.
19. Webb, P., Anderson, C.M., Valentine, C., Nguyen, P., Marimuthu, A., West, B.L., Baxter, J.D., and Kushner, P.J. (2000). The nuclear receptor corepressor (N-CoR) contains three isoleucine motifs (I/LXXII) that serve as receptor interaction domains (IDs). Mol. Endocrinol. *14*, 1976–1985.
20. Ghosh, J.C., Yang, X., Zhang, A., Lambert, M.H., Li, H., Xu, H.E., and Chen, J.D. (2002). Interactions that determine the assembly of a retinoid X receptor/corepressor complex. Proc. Natl. Acad. Sci. USA *99*, 5842–5847.
21. Moraitis, A.N., Giguere, V., and Thompson, C.C. (2002). Novel mechanism of nuclear receptor corepressor interaction dictated by activation function 2 helix determinants. Mol. Cell. Biol. *22*, 6831–6841.
22. Zhang, H., Thomsen, J.S., Johansson, L., Gustafsson, J.A., and Treuter, E. (2000). DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. J. Biol. Chem. *275*, 39855–39859.
23. McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Krones, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., et al. (1998). Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev. *12*, 3357–3368.
24. Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J., and Yamamoto, K.R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. *12*, 3343–3356.
25. Ribeiro, R.C., Apriletti, J.W., Wagner, R.L., Feng, W., Kushner, P.J., Nilsson, S., Scanlan, T.S., West, B.L., Fletterick, R.J., and Baxter, J.D. (1998). X-ray crystallographic and functional studies of thyroid hormone receptor. J. Steroid Biochem. Mol. Biol. *65*, 133–141.
26. Cheskis, B.J., McKenna, N.J., Wong, C.W., Wong, J., Komm, B., Lyttle, C.R., and O'Malley, B.W. (2003). Hierarchical affinities and a bipartite interaction model for estrogen receptor isoforms and full length steroid receptor coactivator (SRC/p160) family members. J. Biol. Chem. *278*, 13271–13277.
27. Northrop, J.P., Nguyen, D., Piplani, S., Olivan, S.E., Kwan, S.T., Go, N.F., Hart, C.P., and Schatz, P.J. (2000). Selection of estrogen receptor beta- and thyroid hormone receptor beta-specific coactivator-mimetic peptides using recombinant peptide libraries. Mol. Endocrinol. *14*, 605–622.
28. Zhang, J., and Lazar, M.A. (2000). The mechanism of action of thyroid hormones. Annu. Rev. Physiol. *62*, 439–466.
29. Katzenellenbogen, B.S., Choi, I., Delage-Mourroux, R., Ediger, T.R., Martini, P.G., Montano, M., Sun, J., Weis, K., and Katzenellenbogen, J.A. (2000). Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J. Steroid Biochem. Mol. Biol. *74*, 279–285.
30. Dutertre, M., and Smith, C.L. (2000). Molecular mechanisms of selective estrogen receptor modulator (SERM) action. J. Pharmacol. Exp. Ther. *295*, 431–437.
31. Osborne, C.K., Zhao, H., and Fuqua, S.A. (2000). Selective estrogen receptor modulators: structure, function, and clinical use. J. Clin. Oncol. *18*, 3172–3186.
32. Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927–937.
33. Bramlett, K.S., Wu, Y., and Burris, T.P. (2001). Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. Mol. Endocrinol. *15*, 909–922.
34. Shang, Y., and Brown, M. (2002). Molecular determinants for the tissue specificity of SERMs. Science *295*, 2465–2468.
35. Feng, W., Ribeiro, R.C., Wagner, R.L., Nguyen, H., Apriletti, J.W., Fletterick, R.J., Baxter, J.D., Kushner, P.J., and West, B.L. (1998). Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science *280*, 1747–1749.
36. Xu, H.E., Lambert, M.H., Montana, V.G., Plunket, K.D., Moore, L.B., Collins, J.L., Oplinger, J.A., Kliewer, S.A., Gampe, R.T., Jr., McKee, D.D., et al. (2001). Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. USA *98*, 13919–13924.
37. Needham, M., Raines, S., McPheat, J., Stacey, C., Ellston, J., Hoare, S., and Parker, M. (2000). Differential interaction of steroid hormone receptors with LXXLL motifs in SRC-1a depends on residues flanking the motif. J. Steroid Biochem. Mol. Biol. *72*, 35–46.
38. Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733–736.
39. Ko, L., Cardona, G.R., Iwasaki, T., Bramlett, K.S., Burris, T.P., and Chin, W.W. (2002). Ser-884 adjacent to the LXXLL motif of coactivator TRBP defines selectivity for ERs and TRs. Mol. Endocrinol. *16*, 128–140.
40. Chang, C., Norris, J.D., Gron, H., Paige, L.A., Hamilton, P.T., Kenan, D.J., Fowlkes, D., and McDonnell, D.P. (1999). Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol. Cell. Biol. *19*, 8226–8239.
41. Norris, J.D., Paige, L.A., Christensen, D.J., Chang, C.Y., Huacani, M.R., Fan, D., Hamilton, P.T., Fowlkes, D.M., and McDonnell, D.P. (1999). Peptide antagonists of the human estrogen receptor. Science *285*, 744–746.
42. Geistlinger, T.R., and Guy, R.K. (2003). Novel selective inhibitors of the interaction of individual nuclear hormone receptors with a mutually shared steroid receptor coactivator 2. J. Am. Chem. Soc. *125*, 6852–6853.
43. Geistlinger, T.R., and Guy, R.K. (2001). An inhibitor of the interaction of thyroid hormone receptor beta and glucocorticoid interacting protein 1. J. Am. Chem. Soc. *123*, 1525–1526.
44. Warnmark, A., Treuter, E., Gustafsson, J.A., Hubbard, R.E., Brzozowski, A.M., and Pike, A.C. (2002). Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. J. Biol. Chem. *277*, 21862–21868.
45. Marshall, H.E., Merchant, K., and Stamler, J.S. (2000). Nitrosation and oxidation in the regulation of gene expression. FASEB J. *14*, 1889–1900.
46. Hanstein, B., Liu, H., Yancisin, M.C., and Brown, M. (1999). Functional analysis of a novel estrogen receptor-beta isoform. Mol. Endocrinol. *13*, 129–137.
47. Nguyen, J.T., Porter, M., Amoui, M., Miller, W.T., Zuckermann, R.N., and Lim, W.A. (2000). Improving SH3 domain ligand selectivity using a non-natural scaffold. Chem. Biol. *7*, 463–473.