Hierarchical Affinities and a Bipartite Interaction Model for Estrogen Receptor Isoforms and Full-length Steroid Receptor Coactivator (SRC/p160) Family Members*

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Nuclear receptor (NR)-mediated transcription is driven by dynamic multiprotein coactivator complexes, the composition of which is thought to determine the biological activity of NRs at specific promoters. The extent to which NRs discriminate between a spectrum of potential binding partners is intuitively a function of the inherent affinities of these individual interactions. Using real time interaction analysis with BIAcore, we evaluated the affinities and kinetics of the interactions of full-length members of the SRC/p160 coactivator family with estrogen receptor α (ERα) and ERβ bound to a variety of ligands. We substantiate that 17β-estradiol enhances the affinity of ER-SRC/p160 interactions, whereas 4(OH)-tamoxifen, raloxifene, and ICI-182,780 inhibit these interactions. We show that a well defined, ER isoform-specific hierarchy governs the association of liganded ERs with full length SRC/p160 family members. Moreover, our data indicate that the interaction affinities of the full-length SRC/p160s with ERs are significantly higher then those of the NR interaction domains of the same coactivators, indicating that portions of coactivator molecules distinct from NR interaction domains might participate in receptor-coactivator complex formation. Finally, the interaction kinetics of SRC/p160s with ERs are consistent with a bipartite model, involving initial rapid formation of an unstable intermediate complex, and a subsequent slower reaction leading to its stabilization. We interpret our results as evidence that hierarchical coactivator interaction affinities are an important source of diversity in NR-mediated signaling and that the complexity of receptor-coactivator cross-talk might be best understood in the context of full-length molecules.

Nuclear receptors (NRs)1 mediate the transcriptional response to a variety of lipophilic ligands and metabolic deriva-

* The abbreviations used are: NR, nuclear receptor; AF-1 and -2, activation function-1 and -2, respectively; CREB, cAMP-response element-binding protein; ER, estrogen receptor; RAC3/SRC-3, receptor-

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205 and CREB-binding protein (15).

Strategies for characterizing receptor-coactivator interactions have relied in large part upon oligopeptide fragments corresponding to minimal coactivator NR interaction domains (16, 17). We have previously shown that NRs interact with isolated NR boxes with low (micromolar) affinity and that mutation of individual SRC/p160 NR boxes does not abrogate ligand-dependent ER-SRC/p160 interactions (15). These data suggest that distinct NR interaction domains can substitute for each other and that regions remote from these motifs also contribute energetically to supplement complex formation (9, 15, 18, 19). Moreover, they indicate that analysis of the interactions of full-length molecules might place measurements of receptor-coactivator affinities in a more informative context.

Mindful of these caveats, we have designed an assay system in which the interaction affinity and kinetics of full-length NRs

**Fig. 1.** Full-length SRC/p160 family members can be directly extracted from oocyte extracts. A, Western blotting analysis of the Xenopus oocyte extracts containing SRC-1, TIF2/SRC-2, and RAC3/SRC-3 with anti-FLAG antibody showing unprogrammed lysate (lane 1), SRC-1 (lane 2), TIF2/SRC-2 (lane 3), and RAC3/SRC-3 (lane 4). The arrow indicates specifically reacting FLAG-tagged protein. The lower band represents a cross-reacting species. B–D, typical SRC/p160 immobilization experiment. Anti-FLAG antibody (8000–10,000 RU) was immobilized in all four flow cells as previously described (36). B, immobilization of SRC-1 in flow cell 1. SRC-1-programmed oocyte extract was injected at a flow rate of 5 µl/min over the anti-FLAG antibody-coated surface. After three injections (total extract volume of 120 µl), 1126 RU of SRC-1 was immobilized. Each extract injection was followed by three injections of 10 µl of 0.05% SDS. The tables next to the sensograms show the flow cell number in which injection takes place (column 1), time after the sensogram started (column 2); relative response, the difference between the refractive index before and after injection (column 3); and injected substance (column 4). C, immobilization of TIF2/SRC-2 in flow cell 2. A single injection of 150 µl of TIF2/SRC-2-programmed oocyte extract over the flow cell was sufficient to immobilize 830 RU of TIF2/SRC-2. D, immobilization of RAC3/SRC-3 in flow cell 3. Three injections (120 µl each) of RAC3/SRC-3-programmed oocyte extract were sufficient to immobilize 971 RU of RAC3/SRC-3.
and coactivators can be evaluated. Intact SRC-1, TIF2/SRC-2, and RAC3/SRC-3 were immobilized on the surface of a BIAcore sensor chip. Coactivator interactions with purified ERα and ERβ were studied over a range of ER concentrations in the absence and presence of the ER ligands 17β-estradiol, 4(OH)-tamoxifen, raloxifene, and ICI-182,780 using real time interaction analysis. Our data indicate that SRC/p160 proteins interact with liganded ERs with affinities higher than those of individual NR interaction domains. They show that ERs and ERβ have robust 17β-estradiol-induced affinity preferences for particular SRC/p160s and that formation of the ER-SRC/p160 complex involves a transitional intermediate.

EXPERIMENTAL PROCEDURES

Equipment and Reagents—The BIAcore 2000 system, sensor chips CM 5 (certified), Tween 20, and amine coupling kit were obtained from BIAcore Inc. (Piscataway, NJ). Purified recombinant human ERα and ERβ were obtained from PanVera Corp. (Madison, WI). 17β-Estradiol and 4(OH)-tamoxifen and FLAG reagents were obtained from Sigma. Raloxifene and ICI-182,780 were synthesized at Wyeth (Radnor, PA).

Plasmids—The construction of the SRC-1 (20), TIF2/SRC-2, and RAC3/SRC-3 (21) Xenopus expression vectors has been described previously.

Synthesis of Full-length Coactivators—N-terminal FLAG fusions of SRC-1, TIPF2/SRC-2, and RAC3/SRC-3 were expressed in Xenopus oocytes by injection of in vitro synthesized mRNA encoding FLAG-tagged SRC/p160s into 1000 stage VI Xenopus oocytes (50 ng of mRNA/oocyte). Oocytes were lysed as previously described (20).

Interaction Analysis—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 dithiothreitol. FLAG protein immobilization on the BIAcore sensor chip surface was carried out as described previously (15, 22). Binding was measured in arbitrary response units (RU). A signal of 1000 RU corresponds to a surface concentration change of ~1 ng/mm² (15). Anti-FLAG antibody was immobilized using an amine coupling kit, typically resulting in the binding of 8000–10,000 RU of antibody. Crude Xenopus lysates containing FLAG-tagged coactivators were then injected at a flow rate of 5 μl/min over a surface coated with immobilized anti-FLAG antibody. Unprogrammed oocyte lysate was used in each run to establish background binding to the FLAG antibody-coated sensor chip and generate a baseline for each individual assay. To determine whether mass transport limitations might affect receptor-coactivator interactions, binding experiments were performed at the following rates: 5, 10, 30, and 50 μl/min. At flow rates of 10 μl/min and above, the values of k_on and k_off determined by analysis of interaction data using BIAevaluation software were independent of flow rates. All subsequent experiments examined coactivator immobilization were performed with a constant buffer flow of 30 μl/min at 25 °C. Samples of receptor were injected across the surface using a sample loop. After the injection plug had passed the surface, complexes were washed with buffer for an additional 1000 s. Following each injection, the surface was regenerated with one 30-μl injection of 0.05% SDS. To remove immobilized FLAG coactivator, a 30-μl injection of 10 mM glycine, pH 2.0, was used. Data were collected at 1 Hz and analyzed using the BIAevaluation program 3.1 (BIAcore, Inc.) on a PC. This program uses a global fitting analysis method for the determination of rate and affinity constants for each interaction. Kinetic rate errors were determined during data analysis in which immobilized coactivator was subjected to multiple interaction cycles with ERα or -β at different receptor concentrations. Five or six binding cycles at different receptor concentrations were used to obtain the affinity and kinetic rates. Each titration experiment was repeated two or three times for each receptor-coactivator combination. Independent experiments were separately analyzed. The differences in affinity and kinetic rates did not exceed 10%. Refractive index differences for the ERs at different protein concentrations were adjusted using Sigma Plot 5.0 software.

RESULTS

Full-length SRC/p160s Can Be Isolated from Xenopus Oocyte Extracts—Western blotting analysis of the Xenopus oocyte extracts expressing SRC-1, TIPF2/SRC-2, and RAC3/SRC-3 is presented in Fig. 1A. We have recently shown that FLAG-tagged SRC-1 expressed in Xenopus oocytes enhanced progesterone receptor transactivation in an in vitro transcription assay (20), indicating that SRC/p160s expressed in this system retain functionality. To evaluate the affinity and kinetics of the interactions between the full-length SRC/p160 family members and ERα and ERβ, we utilized real time interaction analysis with BIAcore. In this technology, one of the interacting molecules is immobilized onto a solid phase, the chip surface, while the interacting partner is injected into the flow cell. When a soluble macromolecule binds to the immobilized species, it leads to an increase in the macromolecule concentration at the sensor surface, generating a change in the refractive index, which is measured by BIAcore. Binding, measured in RU, is recorded in real time, and obtained data can be used to evaluate the kinetics and affinity of macromolecule interaction.

Crude extracts prepared from SRC/p160 mRNA-programmed Xenopus oocytes were directly injected at a flow rate of 5 μl/min over flow cells coated with immobilized anti-FLAG antibody. The binding of SRC-1 (flow cell 1; Fig. 1B), TIPF2/SRC-2 (flow cell 2; Fig. 1C), and RAC3/SRC-3 (flow cell 3; Fig. 1D) to immobilized anti-FLAG antibody in a typical experiment is shown in Fig. 1. Each extract injection was followed by several washes with 10 μl of 0.05% SDS to strip SRC/p160s of any interacting proteins and to remove other proteins that bind nonspecifically to the surface. After washing, 1126 RU of SRC-1 was immobilized in flow cell 1 (Fig. 1B), 830 RU of TIPF2/SRC-2 was immobilized in the flow cell 2 (Fig. 1C), and 971 RU of RAC3/SRC-3 was immobilized in flow cell 3 (Fig. 1D). Flow cell 4 was exposed to unprogrammed Xenopus oocyte extract and used as a control (data not shown). Binding of ERα and ERβ to anti-FLAG chips primed with unprogrammed Xenopus extract was consistently undetectable (data not shown).

Intact ERα and ERβ Interact with Immobilized Full-length SRC/p160s in a Ligand-dependent Manner—The ability of NR agonists to promote the interaction of NRs with coactivators and the ability of antagonists to block this interaction have been firmly established by previous studies. X-ray crystallo-

FIG. 2. Full-length ERα and ERβ interact in a ligand-dependent manner with intact SRC-1. Overlaid sensograms of injections of ERα (A) and ERβ (B) over immobilized SRC-1. Prior to the injection, receptors (250 nm for ERα and 360 nm for ERβ) were preincubated with corresponding ligand (1 μM) for 1 h at room temperature.
Fig. 3. ERα and ERβ interact in a dose-dependent manner with SRC/p160 family members. A, overlaid sensograms of injections of ERα at increasing concentrations (from bottom, 1.12, 4.50, 13.40, 40.0, 120.0, and 360.5 nM) over immobilized SRC-1, TIF2/SRC-2, and RAC3/SRC-3. Prior to each injection, receptor was incubated with 1 μM 17β-estradiol at room temperature for 1 h. B, overlaid sensograms of injections of ERβ at increasing receptor concentrations (from bottom, 3.1, 15.6, 46.8, 140.2, and 420.0 nM) over immobilized SRC-1, TIF2/SRC-2, and RAC3/SRC-3. Prior to each injection, receptor was incubated with 1 μM 17β-estradiol at room temperature for 1 h.
graphic analysis of ERα (23), in addition to receptors for thyroid hormone (9) and 9-cis-retinoic acid (24), as well as peroxisome proliferator-activated receptor-γ (25), have shown this phenomenon to involve agonist- or antagonist-induced realignments of helix 12, respectively potentiating or attenuating AF-2-mediated functions. Accordingly, we first wished to confirm that immobilized SRC/p160s interact in a ligand-dependent fashion with ERα and ERβ under the conditions of our assay. Fig. 2 shows overlaid sensograms of injection of ERα (120 nM) (Fig. 2A) and ERβ (240 nM) (Fig. 2B) in the absence of ligand or the presence of 17β-estradiol, 4(OH)-tamoxifen, raloxifene, and ICI-182,780 over immobilized SRC-1. In their unliganded forms, both receptors interacted with SRC/p160 coactivators. As anticipated, the presence of 17β-estradiol significantly enhanced the binding of both ERs to SRC-1. Similar results were obtained with respect to the interaction of ERα and ERβ with TIF2/SRC-2 and RAC3/SRC-3. Surprisingly, whereas the abrogation of the interaction of ERβ with SRC/p160s in the presence of ICI-182,780, raloxifene, and tamoxifen was essentially complete (Fig. 2B), residual ERα binding in the presence of 4(OH)-tamoxifen (Fig. 2A) was consistently observed. We have previously described the effects of these ligands on ERα and ERβ binding to the NR interaction domain fragments of SRC/p160s (13). In this case, ICI-182,780, raloxifene, and 4(OH)-tamoxifen also reduced binding of both receptors to the coactivator fragments but, as with the full-length SRC/p160s, did not completely abolish this binding.

The ER-SRC/p160 Interaction Fits a Two-step Interaction Model—We next titrated immobilized SRC/p160s with ERα and ERβ in the presence of 17β-estradiol (1 μM) at receptor concentrations ranging from 1.12 to 360.5 nM for ERα (Fig. 3A) and from 3.12 to 420.0 nM for ERβ (Fig. 3B). Saturable interaction was detected for all three SRC/p160 family members, indicating specific binding of ERs to the coactivators. Saturation was also reached for the ERβ-SRC1 interaction.

We then evaluated several possible interaction models for the ER-SRC/p160 binding. Fig. 4 shows the theoretical progress of receptor-coactivator binding following a Langmuir interaction model (Fig. 4A) and a two-state interaction model (Fig. 4A) upon which have been overlaid the experimental data obtained for ERα injection over SRC-1. As can be seen, the obtained data do not fit adequately into a simple Langmuir interaction model (A + B ↔ AB; Fig. 4A) but fit well into a model that describes a two-state reaction (A + B ↔ AB* ↔ AB; Fig. 4B). Kinetic analysis indicates that rapid initial ER bind-
ing \(k_{\text{on}} = 2 - 7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) leads to formation of an unstable transitional intermediate ER-SRC/p160 complex \(k_{\text{off}} = 0.03 - 0.05 \text{ s}^{-1}\), which then assumes a more stable conformation \(k_{\text{off}} = 2 - 5 \times 10^{-4} \text{ s}^{-1}\) at a significantly slower rate \(0.02 - 0.05 \text{ s}^{-1}\). Similar kinetics, consistent with a bipartite reaction model, were observed for all other ER-SRC/p160 combinations (data not shown).

**Selective Recruitment of Full-length SRC/p160 Family Members by ERα and ERβ**—Based on the two-step interaction model, affinity constants \(K_a\) were calculated for interactions between all ER-SRC/p160 combinations. Distinct affinities of ERα and ERβ for individual SRC/p160s were observed, indicating an interaction hierarchy for ERα of RAC3/SRC-3 > SRC-1 > TIF2/SRC-2 and for ERβ of SRC-1 > TIF2/SRC-2 > RAC3/SRC-3. This order of preference recapitulates that determined previously for the interactions between ERs and the NR interaction domain peptides of SRC/p160 family members (15).

Significantly, however, full-length SRC/p160 family members exhibit affinities 3–5-fold in excess of those determined for the NR interaction domains of the corresponding SRC/p160s (see Table I).

**DISCUSSION**

Current models of receptor-coactivator interactions have been based in large part upon interpretations of observations of molecular fragments made in a wide range of assays and experimental conditions. Whereas such studies have gone a long way toward shaping our understanding of mechanistic aspects of coactivator function, they are of more limited use in designing integrated models of the biology of these factors. For this reason, we sought to develop an approach in which kinetic and affinity measurements of NR-coactivator interactions could be made in the context of full-length molecules in a routine, controlled assay.

In this study, we evaluated the affinities and kinetics of the interaction of full-length SRC/p160 family members with ERα and ERβ. Our results provide evidence that a well defined hierarchy governs interactions between ER isoforms and SRC/p160 family members, such that RAC3/SRC-3 is the partner of preference for ERα, and SRC-1 is the preferred interaction partner for ERβ. Factors such as the relative expression levels of coactivators, their subcellular localization, and their post-translational modification status probably modulate the inherent affinities of individual receptors and coactivators along a given signaling axis. These considerations notwithstanding, however, the fact that ER isoforms exhibit hierarchical affinities for potential binding partners is an intriguing facet of their pharmacology and one that will have an important bearing on the development of ER isoform-specific selective ER modulators. It remains to be seen whether such striking discrimination among coactivators is characteristic of other members of the NR superfamily and whether, on the basis of these data, accurate predictions can be made concerning the interactions of these molecules in cells.

The affinities of full-length SRC/p160 interactions with ERs are appreciably higher in our assay than those of the NR interaction domains of the same coactivators (15). At present, the exact reason for the observed difference is unclear, but it probably involves additional contacts made in the context of the complex between full-length coactivator and receptor. It has been consistently demonstrated that molecular determinants distinct from the LXXLL motifs are important influences on the affinity of NR-coactivator interactions (9, 18, 19). Moreover, the involvement of discrete domains in the C terminus of NRs in recruitment of coactivators has been noted in a number of crystallographic studies (25). Taken together with these studies, our results reiterate that models based upon observations of individual NR box-containing peptides, by failing to account for steric aspects of interactions between full-length molecules, afford a less than complete perspective on fundamental aspects of coactivator biology.

This assertion is borne out by our studies of the effect of the ligand on the interaction of ERs with full-length SRC/p160s. In our previous study using coactivator NR interaction domains, 17β-estradiol enhanced the affinity of receptor-NR box interaction, whereas 4(OH)-tamoxifen, raloxifene, and ICI-182,780 inhibited the interaction (15). Intriguingly, the current study showed that this inhibition was much more complete in the context of the full-length coactivators compared with the NR interaction domains. Moreover, 4(OH)-tamoxifen, when bound to ERα, was a less competent inhibitor of ER-SRC/p160 interactions compared with raloxifene and ICI-182,780.

Our data indicate that interactions between full-length ERs and intact SRC/p160s are bipartite, involving an initial, rapid association of a transitional intermediate and a slower terminal phase. The bipartite interaction can be interpreted in at least two ways. First, it may reflect the dynamics sketched by Nolte and co-workers (25), who postulated an initial docking between a glutamate/lysine NR charge clamp and ionic residues N- and C-terminal of the SRC-1/NCoA-1 NR box. This may be followed by an induced fit interaction between the leucine side chains of the NR box and the hydrophobic cavity in AF-2. Conceivably, the fast-slow interaction kinetics we have observed might be generated by an initial rapid compatibility between charged residues, followed by intricate conformational changes accompanying the apposition of two extended hydrophobic surfaces.

An alternative model arising from the bipartite reaction kinetics described here requires consideration of the stoichiometry of the interaction between NRs and SRC/p160s. A model proposed for the interaction of SRC-1 with a heterodimer of the receptors for all-trans-retinoic acid and 9-cis-retinoic acid suggests that binding of tandem NR boxes occurs in a cooperative manner (26). Specifically, binding of the second NR box to the 9-cis-retinoic acid moiety is pursuant to a conformational change arising from the interaction of all-trans-retinoic acid receptor with ligand and recruitment of the first NR box. Whether such a scenario can be accommodated in the context of the interaction of a single SRC/p160 molecule with an ERα homodimer remains to be determined, but it can be envisaged that the slower phase we observed might correspond to the sterically induced binding of a second NR box. Significantly, this model admits a role for AF-1 in participating in coactivator binding, an event suggested by previous studies of several NRs (27, 28).

Evidence accumulated from our own and other laboratories has indicated that coactivators are organized in vitro into large multiprotein complexes (4, 6, 29). In this respect, it could be justifiably argued that our analysis of individually purified proteins might not be directly comparable with the complexities of transcriptional complexes in living cells. Evidence suggests, however, that contacts between NRs and other transcription factors in these complexes are discrete, typically occurring through only one or two adaptor subunits (30, 31). Given that quantitative analysis of the dynamics of such complexes is technically challenging, we reasoned that a logical first step would be to evaluate the interactions between key components of these complexes and proceed to interpret this information in the context of the larger complex.

Several studies have demonstrated the capacity of post-translational modifications of NRs and/or their coactivators to influence cyclicity of molecular interactions at transcriptionally active promoters (32, 33). For example, the acetylation of
an activator of thyroid receptor/SRC-3 by p300/CREB-binding protein neutralizes the positive charges of two lysine residues adjacent to the core LXLL motif and disrupts the association of activator of thyroid receptor/SRC-3 with promoter-bound ERs. In addition, coactivators have been shown to be substrates for several kinases (34, 35), and the notion is emerging that phosphorylation might influence coactivator affinities for NRs and other coregulators. We anticipate that future studies will determine whether the significance with which post-translational modifications have been invest is sustainable in the context of full-length receptor-coactivator interactions on DNA.

In conclusion, we suggest that the discriminatory relationships we have identified may contribute to the combinatorial diversity by which NR-mediated signaling pathways are so clearly characterized.

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