Binding of full-length P160 coactivators to hormone response element-steroid receptor complexes has been difficult to investigate in vitro. Here, we report a new application of our recently described fluorescence anisotropy microplate assay to investigate gate binding and dissociation of full-length steroid receptor coactivator-1a (SRC1a) from full-length estrogen receptor α (ERα) or estrogen receptor β (ERβ) bound to a fluorescent-labeled (fl) estrogen response element (ERE). SRC1a exhibited slightly higher affinity binding to flERE-ERβ than to flERE-ERα. Binding of SRC1a to flERE-ERα and to flERE-ERβ was 17β-estradiol (E2)-dependent and was nearly absent when ICI 182,780, raloxifene, or 4-hydroxytamoxifen were bound to the ERs. SRC1a binds to flERE-E2-ERα and flERE-E2-ERβ complexes with a $t_1/2$ of 15–20 s. Short LXXLL-containing nuclear receptor (NR) box peptides from P160 coactivators competed much better for SRC1a binding to flERE-E2-ER than an NR box peptide from TRAP220. However, ~40–250-fold molar excess of the P160 NR box peptides was required to inhibit SRC1a binding by 50%. This suggests that whereas the NR box region is a primary site of interaction between SRC1a and ERE-E2-ER, additional contacts between the coactivator and the ligand–receptor–DNA complex make substantial contributions to overall affinity. Increasing amounts of NR box peptides greatly enhanced the rate of dissociation of SRC1a from preformed flERE-E2-ER complexes. The data support a model in which coactivator exchange is facilitated by active displacement and is not simply the result of passive dissociation and replacement. It also shows that an isolated coactivator exhibits an inherent capacity for rapid exchange. 

Estrogens regulate the normal growth and differentiation of reproductive tissues, bone, and the nervous system and play an important role in human pathologies such as osteoporosis and breast, uterine, and liver cancer (1–5). Estrogens exert these diverse biological effects by binding to the intracellular proteins, estrogen receptor (ER)α and ERβ (6). ER and other members of the steroid hormone receptor subfamily of nuclear receptors (NRs) share a common domain structure and overall scheme for transcriptional activation (7, 8). In the classical model for ER action, binding of estrogens enables the ER to dimerize and bind to specific canonical DNA sequences termed estrogen response elements (EREs). Transcriptional activation by ER is largely mediated by two interacting activation functions. Activation function 1, in the N-terminal A/B domain is important in ligand-independent transcription and activation function 2 in the C-terminal ligand binding domain is important in ligand-dependent recruitment of coactivators. The bound coactivators help assemble a dynamic, rapidly changing, multiprotein complex that facilitates both chromatin remodeling and formation of an active transcription complex (9–14). When agonists are bound to ER, several ER helices align to form a hydrophobic cleft that is critical for coactivator binding. The long side chains in selective estrogen receptor modulators, such as 4-hydroxytamoxifen (the active metabolite of tamoxifen) and raloxifene (RAL), contact amino acids in helix 12 of the ER ligand binding domain and change the orientation of helix 12 so that helix 12, rather than coactivators, occupies the hydrophobic cleft (15, 16).

The steroid receptor coactivator (SRC) or P160 family of coactivators, consisting of SRC1/NCoA-1, SRC2/GRIP1/TIF2/NCoA2, and SRC3/ACTR/AIB1/p/CIP/RAC3/TRAM1, are perhaps the best known of the many coactivators implicated in ER-mediated transactivation (17, 18). The P160 coactivators interact with the binding cleft in the ER ligand binding domain via highly conserved α-helical Leu-X-Leu-Leu (LXXLL) motifs, also called NR boxes. Most P160 proteins contain a central nuclear receptor-interacting domain made up of three NR boxes arranged in tandem. An alternatively spliced form of SRC1, called SRC1a, contains a fourth NR box at its C terminus (19). Whereas the NR boxes are thought to play a key role in binding of P160 coactivators to ER, amino acid residues flanking this core motif are also important for ER recognition and
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binding specificity (20, 21). In addition, sequences in the N-terminal region of ER play a role in SRC1 binding (22). In vivo, coactivators can interact with diverse proteins including the cointegrators p300/CREB-binding protein and PCAF (23–26). The DRIP/TRAP/ARC multiprotein complex represents a second class of coactivator complex implicated in ER-mediated transactivation. This complex interacts with liganded ER AF2 via two LXXLL motifs in its DRP205/TRAP220 subunit (27).

Chromatin immunoprecipitation results and other data suggest that the components of transcription complexes at EREs undergo rapid exchange and replacement. In a simplified formulation of this sequential model of coregulator exchange, the ER initially recruits an SRC-p300/CREB-binding protein-PCAF complex whose histone acetyltransferase activity acetylates nearby histones, altering the architecture of the promoter regions and enhancing promoter accessibility. The DRIP-TRAP-ARC complex then replaces the SRC-p300/CREB-binding protein-PCAF complex and additional proteins including DNA topoisomerase II, that are linked to the DNA repair machinery, are recruited to the complex prior to steroid receptor activation of transcription (9–11, 13, 14). However, some aspects of the model are difficult to investigate in intact cells, and critical mechanistic questions remain. Addressing questions such as whether components of the complexes can actively displace each other or only enter the complex after the initial components have dissociated, and whether rapid exchange of coactivators is an intrinsic capacity or requires enzymatic modification of the coactivators, requires the development of suitable in vitro approaches.

Previous in vitro studies of ER-P160 coactivator interactions primarily used NR box peptides and NRID-containing protein fragments (28–36). These studies suggest that LXXLL motifs may act only as docking points and that additional contacts outside of the NRID may be required for more complete binding, emphasizing the need to study these interactions with full-length proteins. In addition, because binding of ER to the ERE is thought to precede coactivator binding (37), and the ER also influences ER conformation and the ability of the ER to recruit coactivators (38–45), it is important that ER-coactivator interaction be examined in a context where ER is pre-bound to its DNA binding site.

Perhaps because of instability, the tripartite complex composed of the ERE, ER, and P160 coactivator is not detected using electrophoretic mobility shift assays (data not shown). In contrast to electrophoretic mobility shift assays, surface plasmon resonance and fluorescence anisotropy/fluorescence polarization assays provide real-time analysis of macromolecular interactions. Cheskis and co-workers (29) used surface plasmon resonance to analyze binding of P160 coactivators to liganded ER. However, this important work did not involve a physically isolated P160 coactivator, or ER bound to EREs, and has not stimulated subsequent studies. Recently, we developed the fluorescence anisotropy/polarization microplate assay (FAMA) to analyze the interactions of steroid receptors with their DNA response elements in ultra low-volume microplates (46). In this assay, after excitation with polarized light, the relatively small fluorescein-labeled consensus ERE (flERE) undergoes rotational diffusion more rapidly than the time required for light emission. Therefore, the position of the flERE at the time of emission is largely randomized, resulting in depolarization of most of the emitted light (Fig. 1A). When the ER binds to the flERE, the larger size of the flERE-ER complex causes rotational diffusion to be slower, increasing the likelihood that the flERE-ER complex will be in the same plane at the time of emission as it was at the time of excitation. Therefore, the emitted light will be more highly polarized (Fig. 1B). This change is observed as an increase in fluorescence polarization, or the closely related parameter, fluorescence anisotropy. We also demonstrated that binding of anti-ERα antibodies to the flERE-ERα complex resulted in a further increase in anisotropy, suggesting that the utility of the FAMA could be extended to the study of ERE-ER-coregulator interactions (modeled in Fig. 1C). Results from earlier cuvette-based studies also suggested that the FAMA could be developed for studies of coregulator interactions (47, 48).

Here, we show that the FAMA provides a reliable in vitro assay for monitoring the binding of a full-length P160 coactivator, SRC1a, to flERE-ER complexes and for monitoring the dissociation of flERE-ER-SRC1 complexes. We analyzed the effects of estrogenic ligands and selective estrogen receptor modulators on SRC1 binding and the relative ability of several NR box peptides to block binding of full-length SRC1 to flERE/E2-ERα and flERE/E2-ERβ complexes. The ability of NR box peptides to stimulate dissociation of full-length SRC1a from preformed flERE/E2-ERαSRC1a and flERE/E2-ERβSRC1a complexes suggests that rapid coactivator exchange results from active displacement of the coactivator from the receptor-DNA complex.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Full-length FLAG epitope-tagged human ERs (ERα) and FLAG epitope-tagged human ERβ were expressed in the presence of 200 nm E2, purified in the presence of 20 nM E2, and quantitated as described previously (45, 49). Full-length FLAG epitope-tagged human SRC1a was purified as described (50). Different preparations of SRC1a were used in the several experiments. Optimal binding of SRC1a to flERE-ER complexes was determined from saturation curves done with each preparation of SRC1a (data not shown). Different concentrations of SRC1a are therefore used in some experiments.

The nuclear receptor interaction box 2 (NR-2) and box 4 (NR-4) peptides of SRC1 and the NR-2 peptide of TRAP-220 were synthesized by Abgent (San Diego, CA). The GRIP1 NR-2 peptide was a gift from Dr. Jean Edwards (Baylor College of Medicine, Houston, TX). The amino acid sequences of the NR box peptides are as follows: SRC1 NR box 2, LTERHKILHRLLQE; SRC1 NR box 4, QAQQKSLQQLLTE; SRC2/GRIP1 NR box 2, KHKILHRLLQDSL; TRAP-220 NR box 2, KNHPMLMNLLKDN. The histone methytransferase peptide (HMT2P) was a gift from Dr. Satish Nair (University of Illinois, Urbana, IL). Its sequence is ARTKQTARKSTGGK.

Oligonucleotides—A 30-bp oligonucleotide containing the CERE was synthesized with fluorescein (6-FAM) at its 5′ end using phosphoramidite chemistry and PolyPak II (Glen Research Corp., Sterling, VA) purified by the Biotechnology Center (University of Illinois). The sequence of this fluorescein-labeled sense strand, with the ERE half-sites underlined, is: 5′-
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fl-CTAGATTACGGTCACTGACCTTTACTCA-3’. $A_{260}$ values were measured to calculate the oligonucleotide concentration. The ~60% degree of fluorescein incorporation was determined as described (51). The fluorescein-labeled sense strand was annealed with an equimolar concentration of the unlabeled antisense strand.

Fluorescence Anisotropy Microplate Assays—FAMA was carried out with some modifications of our earlier methods (46). The fluorescein-labeled double-stranded cERE was diluted to 1 nM in the anisotropy buffer (15 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 5% glycerol, 0.05% Nonidet P-40, 2 ng of poly(dl-dC), 100 nM E2, and added to the wells of a black 96-well low-volume, high efficiency HE microplate (Molecular Devices Corp., Sunnyvale, CA). A concentration of human ER identified in preliminary studies as sufficient for saturated binding of human ER to the flERE probe (usually 15 nM) was added to the wells in a total reaction volume of 20 μl. The reactions were mixed by pipetting and incubated at room temperature in the dark. Anisotropy values were measured until the reactions reached equilibrium (usually within 15 min). SRC1α was diluted to appropriate starting concentrations in a buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 0.2 mM EDTA, 15% glycerol, 0.1% Nonidet P-40, and 0.5 mg/ml insulin (or bovine serum albumin). Our ongoing studies showed that the presence of Nonidet P-40 and EDTA had no effect and the Nonidet P-40 could be left out (data not shown). The indicated amounts of SRC1α were then added to wells containing the equilibrated flERE-ER binding reaction. The reactions were mixed by pipetting and incubating at room temperature in the dark. Anisotropy values were measured until the reactions reached equilibrium (within 10 min, see Fig. 4). Anisotropy changes resulting from SRC1α binding were calculated by subtracting the anisotropy values measured for the flERE-ER reactions from the values measured for the flERE-ER-SRC1α reactions. For studies of binding affinity, a percent bound value was calculated at each concentration of SRC1α using the simple expression: (anisotropy change maximum anisotropy change) × 100. After plotting the percent probe bound versus SRC1α concentration, an apparent $K_d$ was determined as the SRC1α concentration at which 50% of the flERE-ER complex was bound to SRC1α. Anisotropy measurements were made on the Ultra Evolution Multifunctional Microplate Reader (Tecan, Research Triangle Park, NC), or on a more sensitive PHERAS-tar High-End Microplate Reader (BMG LabTech Inc., NC). Although outcomes were independent of the instrument used, because of different gain settings on the instruments, the magnitude of the anisotropy changes is not identical in the different figures. Unless otherwise indicated, Sigma Plot was used to fit the data and calculate data from the curves.

Ligand Exchange Experiments—For ligand exchange experiments, a concentration of E2-ER that produces saturated binding to the flERE was added to the anisotropy buffer (plus 15 μg of bovine serum albumin to help stabilize the ERs and 100 nM of the indicated ligand) in the wells of the microplate. Following a 1-h incubation at room temperature, flERE was added to a concentration of 1 nM in a total reaction volume of 20 μl. The reactions were mixed by pipetting and incubated at room temperature. An amount of SRC1α required to achieve ~90–100% of maximum binding of the flERE-ER complex was added and anisotropy was measured as described above.

On Rate Experiments—For on rate experiments, a concentration of E2-ER that produces saturated binding to the flERE was added to the anisotropy buffer containing 1 nM flERE and then incubated for 30 min at room temperature. A near saturating concentration of SRC1α (15 nM) was added to the pre-bound flERE-E2-ER complex in a total reaction volume of 20 μl. Kinetic measurements were carried out at room temperature by taking anisotropy measurements at the indicated time points after SRC1α addition. The anisotropy change when binding reached a plateau at equilibrium was set equal to 100% binding.

Peptide Competition—Peptide competition experiments used ER concentrations that achieved maximal binding of 1 nM flERE in the anisotropy buffer. The indicated amounts of peptide were added to equilibrated flERE-E2-ER binding reactions in a total reaction volume of 20 or 15 μl for the experiments comparing the SRC peptides and the TRAP-220 peptide, respectively, and incubated for 30 min at room temperature in the dark. For competition experiments using peptides derived from SRCs, a concentration of SRC1α that produces ~90–100% of maximal binding of the flERE-ER complex was added. For the TRAP-220 peptide, a concentration of SRC1α that produces ~70–80% of maximal binding of the flERE-ER complex was added. Anisotropy measurements were made as described above. Data for the competition experiments were graphed by curve fitting with Sigma Plot (Figs. 5 and 7A).

Analysis of SRC1α Exchange—Peptide-mediated dissociation experiments used the ER and SRC1α concentrations described above for the peptide competition experiments. Following equilibration, different peptide concentrations were added. The calculated anisotropy change before addition of peptide was set as the maximum anisotropy change at time 0. Kinetic measurements were carried out at room temperature (~22 °C) by taking anisotropy measurements at the indicated time points after peptide addition. The Kaleidagraph® program was used to fit data collected from the NR box peptide and the control HMTP2 peptides with a logarithmic trend line for the NR box peptides and a linear trend line for the HMTP2 peptide. The SRC1 NR box 2 peptide exhibited intrinsic fluorescence that could in principle nonspecifically reduce anisotropy values and calculated anisotropy changes. However, inhibition results remained the same whether or not the data were corrected for background fluorescence of the peptide.

In the exchange experiments it was important to show that dilution was sufficient to nearly abolish rebinding by SRC1α that dissociated from the flERE-E2-ER complexes. In the dilution experiments, 1 nM flERE and 15 nM ER were preincubated for 30 min at room temperature. Two μl of pre-bound flERE-E2-ER complex was diluted 1:10 into anisotropy buffer in a final volume of 20 μl. SRC1α was present or absent in the anisotropy buffer used for dilution. The SRC1α concentration used here equals the final SRC1α concentration used in the active displacement experiments described below after dilution. Kinetic measurements were carried out at room temperature by taking anisotropy measurements at the indicated time points after dilution of the flERE-E2-ER complex.
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For active displacement experiments, the indicated concentration of SRC1α was added to preincubated fERE-E2-ER complex in a total reaction volume of 20 μl. After SRC1α binding reached equilibrium, 2 μl of fERE-E2-ER-SRC1α complex was diluted into 18 μl of anisotropy buffer containing the indicated concentrations of SRC1 NR box 4 or GRIP1 NR box 2 peptide. The anisotropy change at time 0 was set equal to 100% binding. Kinetic measurements were carried out at room temperature by taking anisotropy measurements at the indicated time points after diluting the fERE-E2-ER-SRC1α complex.

RESULTS

FAMA Analysis of the Interaction of Full-length SRC1α with fERE-E2-ER Complexes—Previously, we used the FAMA to analyze binding of steroid hormone receptors and RNA-binding proteins to their response elements in a small sample volume, microplate format (46, 52). To extend the utility of the FAMA, we tested its ability to measure binding of full-length SRC1α to a pre-formed fERE-E2-ER complex. Theoretically, the larger fERE-E2-ER-SRC1α complex should undergo even slower rotational diffusion than the fERE-E2-ER complex, resulting in an anisotropy increase above that seen on binding of E2-ER to the fERE (Fig. 1). Control experiments showed that incubation with SRC1α did not change the basal anisotropy of the fERE probe (data not shown). After prebinding 1 nM fERE with a saturating concentration of liganded ERα or ERβ, SRC1α was added to the indicated concentrations (Fig. 2). Robust binding curves were obtained with apparent Kd values of ~14 and ~6 nM for binding of full-length SRC1α to the fERE-E2-ERα and fERE-E2-ERβ complexes, respectively (Fig. 2). These data demonstrated that despite the substantial size of the initial fERE-E2-ER complexes, we could reliably monitor the binding of SRC1α to these complexes.

Binding of SRC1α to fERE-E2-ER Complexes Is Estrogen-dependent—Studies performed using glutathione S-transferase pull-down experiments, and other techniques, demonstrated that SRC proteins bind to E2-ER, but not to antagonist-bound ER (53). To test whether the SRC1α binding measured by the FAMA was ligand-specific, we analyzed the ligand dependence of SRC1α binding to fERE-E2-ER complexes. First, we carried out in vitro ligand exchange by incubating E2-ER with 100 nM E2, the pure antagonist ICI 182,780, or the selective estrogen receptor modulators, 4-hydroxytamoxifen and RAL. Binding of fERE-E2-ERα and fERE-E2-ERβ with SRC1α was set equal to 100%. When the E2 bound in the ligand binding pocket of ERα or ERβ was replaced with ICI, 4-hydroxytamoxifen, or RAL, there was a nearly complete loss of SRC1α binding to the fERE-ERα and fERE-ERβ complexes (Fig. 3).

SRC1α binds rapidly to fERE-E2-ER Complexes—Studies in intact cells suggest that initial binding of ERα and ERβ to EREs in DNA is quite rapid (54, 55). To determine whether isolated SRC1α also binds rapidly to fERE-E2-ER complexes, we analyzed the initial binding of SRC1α to preformed fERE-E2-ER complexes, respectively, and 98 and 162 mA for ERα and ERβ, respectively.

For active displacement experiments, the indicated concentration of SRC1α was added to preincubated fERE-E2-ER complex in a total reaction volume of 20 μl. After SRC1α binding reached equilibrium, 2 μl of fERE-E2-ER-SRC1α complex was diluted into 18 μl of anisotropy buffer containing the indicated concentrations of SRC1 NR box 4 or GRIP1 NR box 2 peptide. The anisotropy change at time 0 was set equal to 100% binding. Kinetic measurements were carried out at room temperature by taking anisotropy measurements at the indicated time points after diluting the fERE-E2-ER-SRC1α complex.

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complexes. Binding of SRC1a to flERE=E2-ERα and flERE=E2-ERβ complexes was extremely rapid with half-times for binding of 15–20 s for both the ERα and ERβ complexes (Fig. 4, A and B). These data indicate that purified SRC1a, in the absence of any further modifications by enzymes, exhibits the capacity for very rapid binding to ERE-E2-ER complexes.

Role of NR Boxes in SRC1a Binding to ER Complexes—When E2 is bound to ER, the ER ligand binding domain assumes a conformation resulting in a hydrophobic coactivator binding cleft that interacts with the LXXLL motifs in coactivators. Little is known about the relative importance of the LXXLL motifs and amino acid sequences outside of the LXXLL motifs in the interaction of coactivators with ERs. Because coactivator peptides containing LXXLL motifs are quite small relative to the flERE-ER complex, their binding to the flERE-ER complex does not increase the anisotropy signal. This difference in the anisotropy signal upon binding of an LXXLL peptide, or the full-length SRC1a, to flERE-ER allowed us to carry out competition experiments to analyze the role of the NR boxes in binding of SRC1a to ERE-ER complexes.

Previous studies suggested that the interaction of SRC1a with ER may be mediated primarily through NR boxes 2 and 4 (32, 33). We therefore tested the ability of peptides containing either SRC1a NR box 2 or box 4, or SRC2/GRIP1 NR box 2 and their native flanking amino acid sequences, to inhibit binding of full-length SRC1a to flERE=E2-ERα and flERE=E2-ERβ. The concentrations of peptide required to inhibit SRC1a binding to flERE=E2-ERα by 50% (the IC50) were weaker than full-length SRC1a (9, 12, 14). To test aspects of this model using isolated proteins, we determined if the SRC1 NR box peptides, mimicking incoming coactivator complexes, could be used to demonstrate rapid dissociation of pre-formed flERE=E2-ER-SRC1a complexes. For our studies to provide accurate analysis of the loss of SRC1a binding it is important that the ER in the flERE=E2-ER-SRC1a complexes remains on the flERE and does not dissociate over the time course of our experiments. Dilution experiments with flERE=E2-ERα and flERE=E2-ERβ confirmed that these complexes were quite stable and that neither ERα nor ERβ undergoes significant dissociation from the fluorescein-labeled consensus ERE over the time course of our experiments (data not shown).

We tested the dissociation of flERE=E2-ER-SRC1a complexes in the presence of the NR box peptide

**FIGURE 3.** Binding of full-length SRC1a to flERE is estrogen-dependent. In vitro ligand exchange reactions were carried out as detailed under “Experimental Procedures” with 15 nM E2-ERα or E2-ERβ and 100 nM of the indicated ER ligand. Reactions were then incubated with 1 nM flERE at room temperature and anisotropy measured. 25 nM purified, full-length SRC1a was added and the samples were incubated at room temperature. Binding of SRC1a to flERE=E2-ERα and flERE=E2-ERβ was set equal to 100%. The data represent the average of at least three independent experiments ± S.D., with the exception of the flERE-RL-ERα-SRC1a samples, which represent the average of two independent experiments. Maximum actual anisotropy values for flERE-E2-ER and flERE-E2-ER-SRC1a were 70 and 84 mA for E2-ERα, respectively, and 81 and 101 mA for E2-ERβ, respectively. OHT, 4-hydroxytamoxifen.

**FIGURE 4.** SRC1a binds rapidly to flERE-E2-ER complexes. A, 15 nM E2-ERα; or B, E2-ERβ was incubated with 1 nM flERE at room temperature for 30 min. 15 nM SRC1a was added and the anisotropy value was measured. The anisotropy values of binding of SRC1a to the flERE-E2-ERα and flERE-E2-ERβ complexes at t = 300 s was set equal to 100%. The data represent the average of at least three independent experiments. For clarity, error bars are not shown. The actual anisotropy values in panel A at t = 300 s for flERE-E2-ERα and flERE-E2-ERβ were 73 and 96 mA, respectively. For the ERβ complexes in B the anisotropy values were 81 and 116 mA, respectively.
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FIGURE 5. NR box peptides elicit differential inhibition of full-length SRC1a interaction with flERE-ER. 15 nM E$_2$-ER$\alpha$ (A) or E$_2$-ER$\beta$ (B) was incubated with 1 nM flERE for 30 min at room temperature. The indicated concentrations of NR box peptide were added and the samples were incubated for an additional 30 min at room temperature. 25 nM purified, full-length SRC1a was then added and samples were incubated at room temperature and anisotropy measured. Anisotropy changes were calculated by subtracting the anisotropy value measured for each flERE-ER + peptide reaction from the value measured for each flERE-ER + peptide + SRC1a reaction. The anisotropy changes for binding of SRC1a to the flERE-ER$\alpha$ and flERE-ER$\beta$ complexes in the absence of added peptide were set equal to 100%. The data represents the average of at least three independent experiments ± S.D., with the exception of the (flERE-ER$\alpha$ + 1 μM SRC1 NR-2 peptide + SRC1a) samples, which represents the average of two independent experiments. Maximum actual anisotropy values for flERE-ER + peptide and flERE-ER + SRC1 were 60 and 84 mA for the ER$\alpha$ complexes, respectively, and 70 and 104 mA for the ER$\beta$ complexes, respectively.

A

B

FIGURE 6. SRC1a-ERE complexes dissociate rapidly. 15 nM E$_2$-ER$\alpha$ (A) or E$_2$-ER$\beta$ (B) was incubated at room temperature with 1 nM flERE. SRC1a was added to 25 nM and samples were incubated at room temperature and the anisotropy measured. For the flERE-ER$_2$-ER$\alpha$-SRC1a complex, 3 μM SRC1 NR box 2, 8 μM SRC1 NR box 4, and 8 μM HMTP2 were added. For the flERE-ER$_2$-ER$\alpha$-SRC1a complex, 8 μM SRC1 NR box 2, 9 μM SRC1 NR box 4, and 9 μM HMTP2 were added. The anisotropy of each sample was then measured at brief time intervals over the next 25–30 min. The anisotropy changes for binding of SRC1a to the flERE-ER$_2$-ER$\alpha$ and flERE-ER$_2$-ER$\beta$ complexes at t = 0 were set equal to 100%. The data represents the average of at least three independent experiments. For clarity, error bars are not shown. Maximum actual anisotropy values for flERE-ER$_2$ + peptide and flERE-ER$_2$ + SRC1 were 60 and 84 mA for the ER$\alpha$ complexes, respectively, and 70 and 104 mA for the ER$\beta$ complexes, respectively.

concentrations that produced maximum inhibition of SRC1a binding to flERE-ER$_2$-ER in the competition assays shown in Fig. 4. Based on logarithmic trend lines fitted to the data, the times ($t_{1/2}$) required for 50% of the pre-bound SRC1a to dissociate from flERE-ER$_2$-ER were ~1 and ~3 min for the SRC1a NR box 2 and box 4 peptides, respectively (Fig. 6A). The $t_{1/2}$ values for NR box 2- and NR box 4-mediated dissociation of flERE-ER$_2$-ER$\beta$-SRC1a were each ~2 min (Fig. 6B).

TRAP-220 NR Box Peptides Compete with ERE-ER-SRC1a Complexes—Chromatin immunoprecipitation studies indicate that SRC1 that dissociates from the ERE-ER complex would often be replaced by a different coactivator or coactivator complex, such as the DRIP/TRAP/ARC complex (10, 11, 13). We therefore tested the ability of a peptide containing the TRAP-220 NR box 2 sequence to dissociate pre-formed flERE-ER$_2$-ER-SRC1a. Previous studies suggested that TRAP-220 NR box peptides may bind to ERs with much lower affinity than SRC NR box peptides (56). To maximize our ability to detect TRAP-220 NR box peptide-mediated dissociation of flERE-ER$_2$-ER-SRC1a, we used concentrations of full-length SRC1a that resulted in only ~70–80% of maximal binding. Because we had to use lower concentrations of SRC1a to carry out the TRAP-220 NR box competitions than the SRC NR box competitions, the anisotropy changes were smaller. Consequently, the variability in the data from these experiments was somewhat worse than for the studies using the SRC NR box peptides and the precision of the data is somewhat lower.

We first tested the potency and efficacy of TRAP-220 NR box 2 peptide-mediated inhibition in competition experiments. The peptide inhibited binding of SRC1a to both flERE-ER$_2$-ER$\alpha$ and flERE-ER$_2$-ER$\beta$ with a relative IC$_{50}$ of ~40 μM (Fig. 7A). In the same experiment, 4 μM SRC1 NR box 4 peptide inhibited formation of flERE-ER$_2$-ER$\alpha$-SRC1a by ~30% and 3 μM SRC1 NR box 4 peptide inhibited formation of flERE-ER$_2$-ER$\beta$-SRC1a by ~50%, whereas 98 μM HMTP2 did not inhibit formation of the flERE-ER$_2$-ER-SRC1a complexes (data not shown).

When a high concentration (140 μM) of TRAP-220 NR box 2 peptide was present, we observed rapid dissociation of SRC1a from the flERE-ER$_2$-ER complexes. Based on logarithmic trend lines fitted to the data, the $t_{1/2}$ values for dissociation of SRC1a from flERE-ER$_2$-ER$\alpha$ and flERE-ER$_2$-ER$\beta$ were ~2.4 and ~2.9 min, respectively (Fig. 7, B and C).
and is then prevented from re-binding because the NR box peptide, which is present in large excess, occupies the coactivator binding cleft or (ii) the NR box peptide actively displaces the bound SRC1a. The most straightforward way to block re-binding of dissociated SRC1a to the complex, without adding NR box peptide, is to dilute the samples. In this method, diluting the reaction after formation of the fERE-ER-SRC1a complex reduces the potential concentration of free SRC1a in solution to a level so low that very little re-binding will occur (see Fig. 2). To carry out these experiments, we used a sensitive instrument that could carry out anisotropy determinations on diluted 20-μl samples containing only 0.1 nM fERE. As a control, we took pre-diluted fERE-ERα and fERE-ERβ complexes and added a concentration of free SRC1a equivalent to the highest concentration of SRC1a that could be present after diluting the fERE-ER-SRC1a complexes. Adding this concentration of SRC1a to the pre-diluted produced fERE-ER complexes produced little or no increase in anisotropy (Fig. 8, A and B). This shows that there is very little re-binding of dissociated SRC1a at the concentration present in the 10-fold diluted samples.

Although there is more electronic noise in assays carried out using 0.1 nM fERE than in standard assays carried out using 1 nM fERE, the outcomes were clear. In the absence of added NR box peptide, at physiologic salt (150 mM), the half-times for dissociation of SRC1a from the fERE-ERα-SRC1a and fERE-ERβ-SRC1a complexes were ~3 and ~4 min, respectively. Addition of increasing amounts of SRC1 NR box 4 peptide, which exhibited similar IC_{50} values for the fERE-ERα-SRC1a and fERE-ERβ-SRC1a complexes in the competition experiments (Fig. 5), resulted in similar concentration-dependent increases in the rate of SRC1a dissociation from fERE-ERα-SRC1a and fERE-ERβ-SRC1a complexes (Fig. 8, C and D). At 25 μM, the NR box 4 peptide t_{1/2} values for displacement of SRC1a from the fERE-ERα-SRC1a and fERE-ERβ-SRC1a complexes were ~33 and ~59 s, respectively (Fig. 8, C and D). GRIP1 NR box 2 peptide displaced SRC1a from fERE-ERα more potently than from fERE-ERβ (Fig. 5) and displaced SRC1a from the fERE-ERα-SRC1a complexes much more rapidly than from fERE-ERβ-SRC1a complexes (Fig. 8, E and F). At 2.5 μM, the GRIP1 NR box 2 peptide t_{1/2} value for displacement of SRC1a from the fERE-ERα-SRC1a was ~12 s whereas at 3 μM GRIP1 NR box 2 peptide displaced SRC1a from the fERE-ERβ-SRC1a complex with a t_{1/2} of ~135 s (Fig. 8, E and F). High NR peptide concentrations increased the rate of dissociation of SRC1a by 5–15-fold compared with dissociation of SRC1a in the absence of NR peptide. This work suggests that the competition data of Fig. 5 is due to displacement of the bound coactivator by the NR box peptides and that active displacement plays an important role in rapid coactivator exchange.

**DISCUSSION**

*Application of the FAMA to Coactivator Binding and High Throughput Screening*—Ligand-dependent recruitment of coactivators to ER is a vital step in ER-regulated gene expression and has therefore been the focus of many recent studies. It is widely accepted that sequences in the coactivators outside of the LXXLL motifs, and the sequence of the hormone response element to which the receptor is bound are important in coactivator binding (20, 21, 38–44, 57). However, the complexity of assays involving DNA binding sites, full-length coactivators, and full-length ER has led nearly all researchers to focus on studies that involve LXXLL peptides, or the receptor interact-
ing domain of P160 coactivators and ER that is not bound to DNA (28–36). Here we describe a microplate-based assay for analyzing interaction of full-length coactivators with steroid hormone receptors bound to fluorescein-labeled DNA binding sites. Because these assays are carried out in low volumes (20 μl), they conserve limited supplies of full-length coactivator.

The important roles of estrogen bound to ER in diseases ranging from breast cancer to osteoporosis has made coactivator binding an attractive target for high throughput screening to identify modulators of ER-coactivator interaction.

Classically, these studies involved identification of ER ligands that bind in the ER ligand binding pocket and result in altered interaction with coactivator LXXLL peptides or the receptor interacting domain, or used cell-based screens. We demonstrate here that in vitro ligand exchange using 4-hydroxytamoxifen, RAL, and ICI 182,780 (Flutamide) is highly effective and is readily combined with FAMA to quantitate coactivator binding (Fig. 3). Ligand exchange followed by FAMA should allow rapid screening of small molecules that bind in the ERs ligand binding pocket and influence coactivator binding. This system should therefore find broad application in screens to identify novel receptor antagonists and agonists.

Many recent studies have focused not on the identification of new receptor ligands, but on the identification of peptides, peptidomimetics, and small molecules that disrupt receptor-coactivator interactions (20, 58–68). Since the changes in anisotropy we measure in the FAMA result from changes in the size of the complex (Fig. 1), binding of the relatively large full-length P160 coactivator, SRC1a, to flERE/ER complex elicits a substantial increase in anisotropy, whereas binding of small peptides and the SRC1 RID fragment do not produce a significant increase in anisotropy (data not shown). With its low volume and microplate format, the FAMA is therefore well suited to high throughput screening of small molecule libraries to identify novel inhibitors of coactivator binding. Our demonstration that NR box peptides compete with SRC1a for binding
to the flERE-ER complex (Figs. 4–7) provides proof-of-principle for this use of the assay.

**Binding of SRC1α to flERE-ER Complexes**—In our binding studies, full-length SRC1α bound saturated flERE-E2-ERβ complexes with an ~2-fold higher affinity than the flERE-E2-ERα complexes. Using surface plasmon resonance, Cheskis and co-workers (29) examined the interaction between ERs (not bound to an ERE) and full-length SRC1. They did not observe much difference in affinity between binding of SRC1 to ERα and ERβ. Because these studies involved immobilized SRC1 purified directly on the support and were done in the absence of the ERE, the fluorescence anisotropy and surface plasmon resonance results are not directly comparable.

Intracellular studies using fluorescence photobleaching and other techniques to probe the binding of ER to DNA suggest that ER moves around rapidly on DNA (54, 55). Chromatin immunoprecipitation and other studies led to the concept of rapid coactivator exchange (9–11, 13, 14). We evaluated SRC1α binding to ERE-ER complexes in a defined system in which only the ERE, purified ER, and the coactivator were present. Our assays do not contain an energy source and there are no known enzyme activities that might covalently modify the baculovirus expressed, purified SRC1α, or the ERs. SRC1α bound very rapidly to the flERE-ER complexes with a t1/2 for binding of 15–20 s. Very rapid binding was also observed by Cheskis and co-workers (29) who used surface plasmon resonance to analyze binding of SRC1 to ERα and ERβ in the absence of the ERE (29). These studies suggest that once the ER binds an estrogen, initial binding of P160 coactivators is extremely rapid. Whether the ER binds the P160 coactivator after binding to an ERE, or before binding to an ERE, may depend on the rate at which E2-ER locates and binds to ERs compared with the rate at which it binds P160 coactivators.

**Role of LXXLL Motifs in SRC1α Binding**—Studies using diverse techniques led to the identification of LXXLL motifs as important in coactivator binding (15, 57, 65, 69–72). However, the contribution of individual LXXLL motifs to binding of full-length P160 coactivators is not well defined. To explore this, we evaluated the ability of several NR box peptides to block binding of full-length SRC1α to the flERE-E2-ER complex. At high concentrations, individual NR box peptides were sufficient to largely abolish binding of SRC1α to the flERE-E2-ER complexes. These data are consistent with the view that the LXXLL-containing NR boxes are necessary for SRC1α binding. However, an ~40–250-fold molar excess of LXXLL peptide over SRC1α was required to inhibit binding of SRC1α to the flERE-E2-ER complexes by 50%. The large molar excess of each NR box peptide that was required to inhibit SRC1α binding was especially striking because the NR box peptides were preincubated with the flERE-E2-ER complexes before adding SRC1α. Our data therefore indicates that individual LXXLL motifs are critical for SRC1α binding, but they are insufficient for high affinity binding of SRC1α to the E2-ERE-ER complex. Therefore, interaction surfaces on SRC1α and ER distinct from the LXXLL motifs in SRC1α and the LXXLL binding cleft in ER make significant contributions to the high affinity with which full-length SRC1α binds to the ERs.

The peptide competition assays allowed exploration of SRC NR box selectivity for ER subtypes using a full-length SRC1α. Our selectivity data are generally consistent with binding studies that determined selectivity by measuring the Kd values of the individual P160 NR box peptides for E2-ERα and E2-ERβ (30, 32, 33, 35). Thus, the SRC NR box selectivity for ER established using NR box peptides seems to hold true even in the context of full-length coactivator. Interestingly, these differences extend to the rate at which NR box peptides displace SRC1α from ERα and ERβ. The GRIP NR box 2 peptide displaces SRC1α from flERE-E2-ERα more than 10 times faster than it displaces SRC1α from flERE-E2-ERβ (Fig. 8, E and F). It has been proposed that NR box selectivity helps define ER-coactivator selectivity (19, 36, 56, 69). The different NR box preferences we, and others, have observed for ERα and ERβ may help explain their different transcriptional activities in target cells despite similar ligand and DNA binding affinities.

We also performed competition assays to evaluate the relative potency of the TRAP-220 NR box 2 peptide. Warnmark et al. (56) reported that ER binds SRC2/GRIP1/TIF2 NR box peptides with much higher affinity than TRAP-220 peptides and that TRAP-220 displays a strong preference for binding to ERβ. In contrast, Burakov et al. (73) found that ER binds to an NR box-containing TRAP-220 fragment with only slightly lower affinity than the NR box-containing SRC1 fragment and that TRAP-220 binds to both ERα and ERβ equally well. We found that the TRAP-220 LXXLL peptide exhibited similar potency in blocking SRC1α binding to flERE-ERα and flERE-ERβ. However, the TRAP-220 NR box peptide is a much weaker competitor than the SRC NR box peptides. The SRC1α NR box 4 peptide is >5-fold more effective than the TRAP-220 peptide in inhibiting SRC1α binding.

**LXXLL Peptides Actively Displace SRC1α from ERE-ER Complexes**—It is widely accepted that the process of regulated transcription initiation by steroid/nuclear receptors involves a complex series of events in which numerous proteins interact with and dissociate from the complex (14). However, studies in intact cells have not readily addressed two important issues. (i) Do coactivators leave the complex by simply dissociating from the complex, after which they are replaced by other coregulators that occupy the now empty coactivator binding site, or are the coactivators actively displaced from the complex by incoming proteins? (ii) The process of coactivator exchange in cells is complex and involves both macromolecular interactions and diverse covalent modifications of the coactivators by several enzymes in the complex. Whether the very rapid replacement of coregulators is due at least in part to intrinsic properties of the coregulators, or only to alterations in protein-protein interactions resulting from covalent modification is unclear. Our studies have begun to address these issues. In the absence of any external agent, the flERE-E2-SRC1α complex does not exhibit the very rapid loss of coregulator seen in intact cells. Studies with two NR box peptides show that the NR box-containing peptides can actively displace full-length SRC1α from the complex and produce rapid exchange. This rapid exchange is consistent with the very rapid dissociation of SRC proteins from ERα and ERβ seen in intracellular systems (54, 55). This data suggests that binding of SRC1α to ER is a dynamic process...
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in which the diverse contacts that contribute to high affinity binding are rapidly and repeatedly made and broken. Even though SRC1a is bound to the ERE-ER complex, this process of partial dissociation, or coactivator breathing, frequently exposes the coactivator binding cleft. When no other coactivator or LXXLL peptide with high affinity for this site is present, SRC1a, which is tethered to the ER through other contacts and is therefore in both high local concentration and close proximity to the coactivator binding cleft, rapidly rebinds to that segment of the ER. When the coactivator peptide is present and coactivator breathing exposes the coactivator binding cleft, the LXXLL peptide is able to enter the coactivator binding cleft. This prevents rebinding by the corresponding LXXLL-containing segment of the SRC1a and therefore favors further dissociation of SRC1a from the ER. As the concentration of LXXLL peptide increases, there is an increasing probability that it will occupy the binding cleft rather than allowing the dissociated segment of SRC1a to rebind.

Covalent modification of coregulators doubtless plays a key role in altering their protein-protein interactions and association with multiprotein complexes on DNA (12, 14). The high affinity of SRC1a for ERE-ER complexes we (Fig. 2) and others (29) report raises the possibility that coregulator breathing, rapidly rebinds to that segment of the SRC1a and therefore favors further dissociation of SRC1a from the ER. As the concentration of LXXLL peptide increases, there is an increasing probability that it will occupy the binding cleft rather than allowing the dissociated segment of SRC1a to rebind.

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