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The Human Estrogen Receptor α Dimer Binds a Single SRC-1 Coactivator Molecule with an Affinity Dictated by Agonist Structure

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Nuclear receptors act as ligand-inducible transcription factors. Agonist binding leads to interaction with coactivator proteins, and to the assembly of the general transcription machinery. In addition to structural information, a thorough understanding of transcriptional activation by the nuclear receptors requires the characterization of the thermodynamic parameters governing these protein/protein interactions. In this study we have quantitatively characterized the interactions of full-length baculovirus expressed human estrogen receptor α (ERα), as well as ERα hormone binding domain (ERHBD) with a fragment of the coactivator protein SRC-1 (amino acid residues 570 to 780). Fluorescence anisotropy and fluorescence correlation spectroscopy of fluorescently labeled $SRC\text{-}1_{570\text{-}780}$ demonstrate unambiguously that the stoichiometry of the SRC-1/ERα/estradiol complex is one coactivator molecule per ERα dimer. The affinity of the estradiol or estriol bound ERα/SRC-1 complexes was found to be significantly higher than that observed in the presence of estrone. No binding was observed in the absence of ligand or in the presence of antagonists. Distinct anisotropy values for the ERα-SRC-1 complexes with different agonists suggest distinct conformations of the complexes depending upon agonist structure.

Keywords: estrogen receptor; coactivator; fluorescence; affinity; photon counting histogram

Introduction

Hormonal regulation of gene activity is mediated in part by nuclear receptors acting as ligand-activated transcription factors (Mangelsdorf *et al.*, 1995). These proteins interact with the ligand, the DNA-response element and, in an agonist-dependant manner, with several proteins acting as transcriptional coactivators, such as CBP/p300, and the p160 family proteins (SRC-1, TIF-2, and SRC-3) (Robyr *et al.*, 2000). These protein/protein interactions are mediated by means of two activation functions (AFs), AF-1 located in the N-terminal region of the receptor, and AF-2 located in the hormone-binding domain. The crystal structures of the ligand-binding domains of several NR (reviewed by Moras & Gronemeyer, 1998) have shown the structural role of helix 12 in the active/inactive switching of the AF-2. In the agonist bound complexes, H12 creates a hydrophobic groove recognized by the coactivator and in contrast, in the antagonist bound complexes, H12 is displaced outward, disrupting this interaction surface.

Specific motifs in the coactivators with the consensus sequence LXXLL called NR-boxes, are the dominant factors in modulating their binding to the NR, as shown by mutational mapping studies, yeast two-hybrid, and GST-pull down interaction experiments (Ding *et al.*, 1998; Feng *et al.*, 1998; Heery *et al.*, 1997; Le Douarin *et al.*, 1996; Mak *et al.*, 1999). SRC-1a contains four NR-boxes. Three of

Abbreviations used: AF, activation function; NR, nuclear receptor; ERHBD, ERα hormone binding domain; FCS, fluorescence correlation spectroscopy; PCH, photon counting histogram; ER, estrogen receptor; E1, estrone; E2, 17 β-estradiol; E3, estriol.

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these (NR-boxes 1-3) are located near the center of the protein, and the fourth, which is lacking in TIF-2, is found in the C-terminal extremity (Heery *et al*., 1997; Kalkhoven *et al*., 1998). Disruption of NR-box 2 has the most profound effect on interaction with ERHBD (Mak *et al*., 1999).

Mutational studies on the NR-boxes (Tremblay *et al*., 1999; Voegel *et al*., 1998), allosteric effects between heterodimers (McInerney *et al*., 1998; Westin *et al*., 1998), as well as ligand dissociation kinetics (Gee *et al*., 1999), suggest a stoichiometry of one coactivator per nuclear receptor dimer. On the other hand, Leers *et al*. (1998) suggest that two TIF-2 molecules could be present on a RXR/TR heterodimer using electrophoretic mobility shift assays. Two structures of complexes between agonist-bound NR ligand-binding domains and NR-box peptides have been solved by X-ray diffraction, in which each HBD monomer is bound by a coactivator derived peptide (Darimont *et al*., 1998; Shiau *et al*., 1998). In addition, a complex between PPARγ HBD and a 88 amino acid residue polypeptide comprising the NR-boxes 1 and 2 from SRC-1 shows each of the two NR-box interacting domains lying in the hydrophobic groove of each HBD monomer (Nolte *et al*., 1998). This argues for a one SRC/NR dimer stoichiometry, although the rest of the SRC-1 molecule is not resolved in the structure.

Despite these studies, no direct determination of the stoichiometry of the NR/coactivator complexes has been carried out in solution under equilibrium conditions. Nor has SRC-1 and NR concentration dependence of the stoichiometry been ascertained. Affinities for NR/coactivators interactions between 1 nM and 770 nM have been estimated from non-equilibrium or relatively indirect methods (Biacore (Suen *et al*., 1998), antibody-mediated fluorescence energy transfer (Zhou *et al*., 1998), and ligand dissociation kinetics (Gee *et al*., 1999)). Here, we have determined unambiguously the stoichiometry of the complexes between SRC-$1_{570\text{-}780}$ and ERHBD or full length ERα over a broad range of concentrations using fluorescence correlation spectroscopy, while fluorescence anisotropy-based titrations were used to determine the affinity of the complexes. We also address the modulation of the interaction energies of these complexes by antagonists and by the three naturally occurring estrogens, i.e. 17-β estradiol and two of its metabolites:estrone and estriol.

Results

Ligand-dependent *in vitro* interaction of GST-SRC-1 with ERE-bound ERα

To study the interaction of SRC-1 with ERα bound to DNA, we first used a modified version of the GST-pull-down assay using GST-SRC-1, ERα expressed in COS cells and a ^{32}P-labeled oligonucleotide containing a consensus ERE. Binding of the labeled ERE to GST-SRC-1 was receptor-mediated since no specific binding was observed using mock transfected COS cell extracts (not shown). Formation of the ERE/ERα/SRC-1 ternary complex increased (more than 20-fold) in the presence of E2 (Figure 1(a)). The EC_{50} (0.4 nM) corresponded to the affinity of the ligand for the receptor previously described by Anstead *et al*. (1997).

Binding was specific for ERα agonists since ERE retention of the ERα:ERE complex onto GST-SRC-1 was observed with natural or synthetic estrogens but not in the presence of various antagonists such as 4-hydroxy-tamoxifen, raloxifen or ICI 182780 (Figure 1(b)). However, it should be noted, as previously reported for the binding of ERα to hTIF1α,

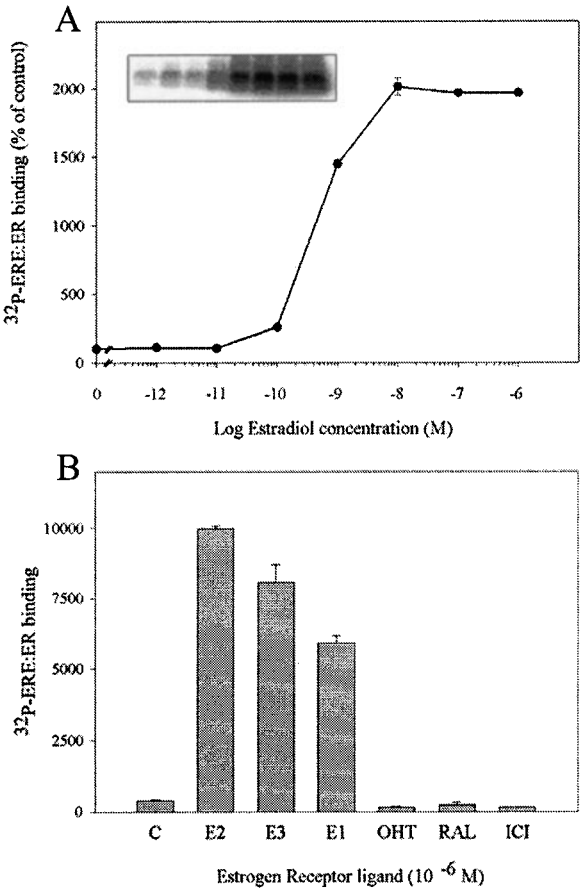

Figure 1. *In vitro* interaction of GST-SRC-1 with ERE-bound ERα. (a) Ligand-dependent interaction of GST-SRC-1 with ERα bound onto DNA. The effect of increasing concentrations of E2 (pM to μM) on the interaction of GST-SRC-1 with ERα in the presence of ^{32}P-labeled ERE was analyzed as described in Material and Methods. Ternary complex formation was measured by β counting or by gel electrophoresis and detection using a Phosphorimager (inset). (b) Ligand specificity of the interaction. Binding was analyzed as in (a) in the absence of ligand (C) or in the presence of 1 μM E2, E3, E1, OHT, Raloxifen (RAL) or ICI 182 780 (ICI). Similar amounts of GST-SRC-1 fusion protein were used as judged by Coomassie blue staining of the gel prior to fluorography. Results represent mean of three determinations ± SD.

that E2 was the most potent ligand in inducing the interaction whereas estrone exhibited a lower efficiency (Thénot *et al.*, 1999).

Characterization of SRC-1/Estrogen Receptor binding with an Alexa488 labeled SRC-1

The interaction of SRC-$1_{570\text{-}780}$ with ERα and ERHBD was next monitored by measuring changes in the steady state fluorescence anisotropy of a fluorescent probe (Alexa 488) covalently coupled to the N terminus of the SRC-1 fragment (^{A488}SRC-1). The anisotropy value is the intensity normalized difference between vertically and horizontally polarized fluorescence emission and is inversely proportional to the rotational diffusion, thus the larger the molecule, the higher the anisotropy (Lakowicz, 1991).

Interaction between ^{A488}SRC-1 and ERHBD or full length ERα is observed as an increase in anisotropy only in the presence of the ERα agonists, but not in the absence of hormone, or in the presence of ERα antagonists (Ral, OH-Tam) (Figure 2(a) and (b)). Moreover, this interaction is reversed by the addition of excess unlabeled SRC-1, which competes for receptor binding (Figure 2(a) and (b)). Identical titration results for SRC-1 labeled with Alexa 568, which differs from Alexa 488 by two additional aromatics rings (Panchuk-Voloshina *et al.*, 1999), demonstrated that the nature of the probe does not influence the binding (data not shown). As in a previous work with TIF-1α (Thénot *et al.*, 1999), titration of a fluorescein-labeled ERE/ERα complex with SRC-1 also revealed an agonist-dependent increase in anisotropy, whereas no change was observed in the absence of ligand or in the presence of antagonist (data not shown).

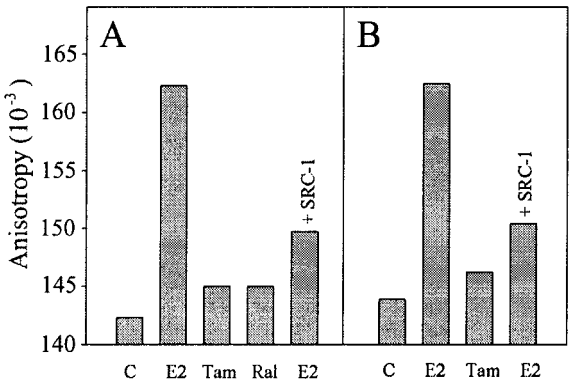

Figure 2. Characterization of the interaction between ERα and ^{A488}SRC-1. (a) Interaction between ^{A488}SRC-1 (5 nM) and ERHBD (100 nM) in the absence of ligand (C) or in the presence of agonists (E2) or antagonists (Ral, Tam). E2-induced interaction is reversed by unlabeled SRC-1 (1 μM). (b) Interaction between ^{A488}SRC-1 (5 nM) and ERα (100 nM). E2-induced interaction is reversed by unlabeled SRC-1 (1 μM).

Determination of the stoichiometry of the SRC-1/ER interaction

Oligomeric state of the ERα

Quantitative analysis of anisotropy binding profiles for the ER/SRC-1 interaction require the unambiguous determination of the final stoichiometry of the complex. First, the oligomeric state of the ERα (full-length and HBD) was characterized in the micromolar concentration range. Quasi elastic light scattering experiments yielded an apparent diameter of 7.5 nm in the absence and presence of estradiol, corresponding to molecular mass values of 60 and 64(±12) kDa, consistent in both cases with a dimer. Cooperative anisotropy-based DNA binding profiles for full length ERα are indicative of a dimerization constant near 10 nM (Boyer *et al.*, 2000).

Stoichiometric titration

Stoichiometric titrations of ^{A488}SRC-1 by ERHBD were performed and the increase in the fluorescence anisotropy of the labeled SRC-1 was measured. Unlabeled SRC-1 was used at a concentration of 880 nM (greater than tenfold K_D for the E2-induced interaction, see below), in the presence of a low concentration of labeled ^{A488}SRC-1 (5 nM) and titrated by ERHBD (Figure 3) in the presence of E2. The anisotropy value is observed to continue to increase well beyond a ratio of one ERHBD monomer per SRC-1 molecule, supporting the hypothesis that the complex stoichiometry is likely two ERHBD monomers/SRC-1. In addition to the stoichiometric titration, a number of other fluorescence-based approaches (data not shown) provided support for the hypothesis that only one SRC-1 fragment is present in complexes with ERα dimers. A fluorescence energy transfer experiment, using ^{A488}SRC-1 (50 nM) as a donor and ^{A568}SRC-1 (50 nM) as acceptor ($R_0 = 60$ Å) revealed no detectable FRET upon addition of ERHBD and E2. Moreover, in the presence of saturating ERHBD and E2, addition of an anti-Alexa 488 antibody lead to a further increase in the anisotropy of ^{A488}SRC-1 whereas no anisotropy supershift was observed for the ^{A568}SRC-1 anisotropy. Finally, in a variation of a classical GST-pull down experiment, no fluorescence from ^{A488}SRC-1 was detected upon mixing with SRC-1-GST proteins loaded on Sepharose beads in the presence of ERα and E2 under conditions of complex formation.

Fluorescence correlation spectroscopy

Although these experiments strongly support a stoichiometry of one SRC-1 molecule per ERα dimer, we sought to perform a direct measure of complex stoichiometry using fluorescence correlation spectroscopy (FCS) with photon counting histogram (PCH) analysis. In an FCS experiment, the distribution of photon counts emanating from

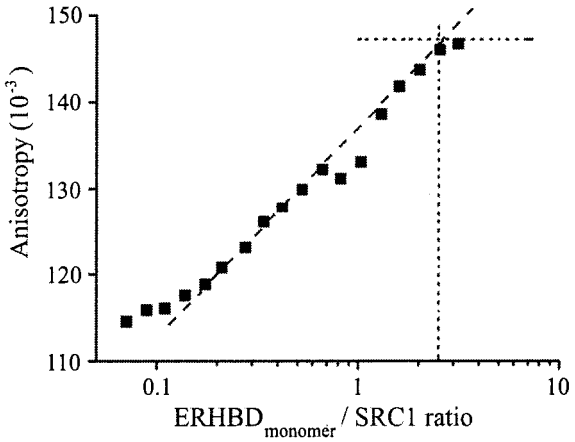

Figure 3. Stoichiometric titration of ^{A488}SRC-1 by ERHBD. Stoichiometric titration of SRC-1 (880 nM) and Alexa 488-SRC-1 (5 nM) by ER-HBD, in the presence of E2 (10 μM). Anisotropy value is observed to increase well beyond a 1/1 [ERHBD]/[SRC-1] ratio.

a small excitation volume (achieved by two-photon excitation in an inverted microscope with a high numerical aperture objective) is described by the individual molecular brightness of the fluorescent molecules and their concentrations (Chen *et al*., 1999a,b). For example, proteins with either one or two dye labels can be successfully resolved by a single measurement (Muller *et al*., 2000).

FCS experiments were performed as described in Materials and Methods using a high nominal concentration of ^{A488}SRC-1 (200 nM) in order to favor a putative 2:1 SRC-1:ERα dimer complex. We note that the concentration of ^{A488}SRC-1 was found by FCS to be threefold lower than the nominal concentration based on the Lowry method. This low value was subsequently shown to be due to loss of the SRC1 molecules which adhere to the walls of the LabTek chamber, since we observed a decrease of intensity with time, which can be recovered by mixing the solution. Repetition of the experiments yielded the correct nominal concentration shortly after pipetting the sample, but this value tended to decrease with time. We note that this phenomenon only occurs in the FCS experiments due to the nature of the chamber, and did not occur for measurements using glass tubes, i.e. the fluorescence anisotropy experiments. We also note that the molecular brightness value from which we deduce the stoichiometry is an absolute value and does not depend upon the concentration of fluorophore.

PCH analysis of the photon count distributions (Figure 4) obtained for SRC-1 alone and with increasing concentrations of ERα (up to 105 nM dimer) in the presence of estradiol fit well to a single species model with a molecular brightness value of 42,600 (±300) cpsm (counts per second and molecule) and a global reduced χ^2 of 1.2 (Table 1). This brightness value corresponds to that of a single Alexa 488 molecule. Dilution of ^{A488}SRC-1 alone over a concentration range of 150 nM to 9 nM ^{A488}SRC-1 yielded a constant value for the molecular brightness (data not shown), demonstrating that no association/dissociation process for the ^{A488}SRC-1 occurs over this concentration range. Since the labeling ratio was determined to be one dye molecule/SRC-1 molecule (see Materials and Methods), and the molecular brightness observed corresponds to that of free Alexa 488, we conclude that ^{A488}SRC-1 is monomeric under these conditions. The fact that the molecular brightness value of the monomeric ^{A488}SRC-1 does not increase upon addition of ERα indicates that SRC-1 is present as a monomer in the complexes with ERα under these conditions. An observed decrease in the diffusion coefficient of the ^{A488}SRC-1 from 90 to 50 $\mu m^2\ s^{-1}$ upon addition of ERα, as well as fluorescence anisotropy measurements carried out in parallel confirm that binding of the ^{A488}SRC-1 to the ERα did indeed occur. Moreover, neither the fluorescence intensity of ^{A488}SRC-1, nor its fluorescence lifetime decreased upon titration with ERHBD or full-length ERα, ruling out the masking of the existence of a 2:1 SRC-1/ER complex through quenching by homotransfer.

Simulations of fractional complex populations

In order to reach an unambiguous conclusion as to the stoichiometry of SRC-1 complexes with ERα, it is necessary to demonstrate that a putative 2:1

Table 1. Molecular brightness and number of particles

ERα (nM)	ε (cpsm)	*n*	χ^2
0	42660	3.97	0.99
5	42660	4.04	1.28
10.5	42660	4.17	1.11
21	42660	4.21	1.71
42	42660	4.19	1.05
84	42660	4.11	0.97
105	42660	3.87	1.16

Molecular brightness (ε) and average number of particles (*n*) of ^{A488}SRC-1 (200 nM nominal) as a function of ERα dimer concentration, in the presence of estradiol (1 μM). Data were fit globally with a single brightness to a single species model. The global reduced χ^2 is 1.2 and the reduced χ^2 of each individual data set is indicated (see the text for discussion).

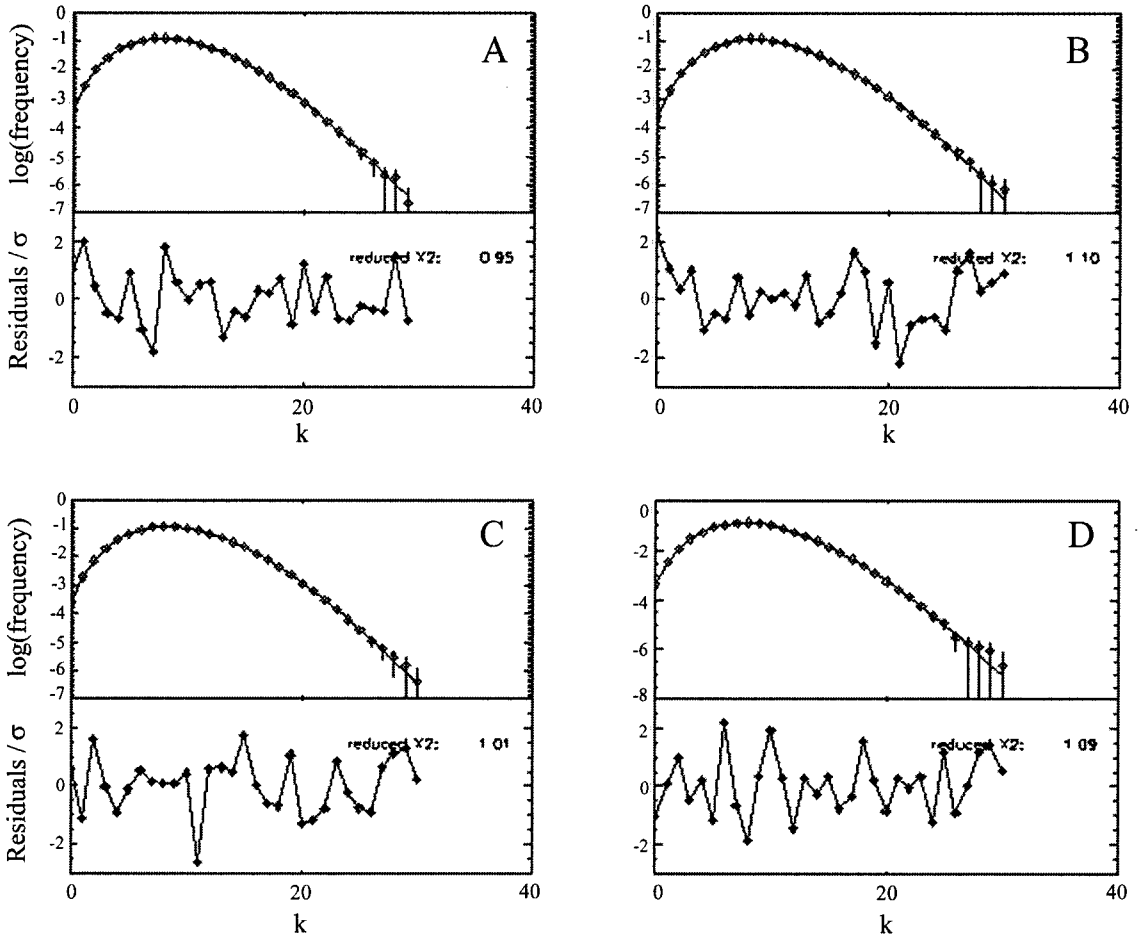

Figure 4. PCH of ^{A488}SRC-1 upon titration by ERα. Photon counting histograms of SRC-1 Alexa (200 nM) in the presence of estradiol, with (a) 0 nM, (b) 10.5 nM, (c) 42 nM or (d) 105 nM, full-length ERα dimer. Data (including values at 5, 21, and 84 nM (not shown)) were fit globally with a single species model, leading to values found in Table 1. The upper panels shows the PCH raw data and the fit, and the lower panels are a plot of the residuals.

complex would have been populated under the conditions tested, and detected with the FCS. In accordance, we have carried out simulations of the fractional population of the 1:1 and 2:1 complexes under different conditions. First, we assumed the presence of two binding sites of equal intrinsic affinity for SRC-1 on the ERα dimer. Since the apparent dissociation constant for the interaction between SRC-1 and ERα dimer estimated from the binding curves (see below) is 30 nM, for the case of two identical binding sites, due to the statistical entropy term, the intrinsic dissociation constant would be 60 nM. In this case, at 70 nM SRC-1 (concentration estimated from the FCS experiments due to loss of protein through adhesion), the 2:1 species would be maximally populated at a level of 9 % at 105 nM ERα dimer.

However, this model considers SRC-1 as a monovalent ligand. A more realistic model (arising from the structure of the PPAR/SRC-1 complex (Nolte *et al.*, 1998)) takes into account the fact that a single SRC-1 molecule can bind both monomers of the ERα dimer, since the SRC-1 fragment contains three LXXLL motifs. In this case, there is no difference in the entropy contribution to the statistics for binding the first and second SRC-1 molecules, and thus the intrinsic and statistical dissociation constants are equal (30 nM). According to this model we would expect the 2:1 species to be populated at a level of 25 % at 70 nM SRC-1 and 105 nM ERα dimer.

In order to reject the hypothesis of this 2:1 species being present we re-analyzed the FCS data at 105 nM ERα and 70 nM SRC-1 (histogram from Figure 4(d)) assuming the same brightness for the 1:1 species and the free ^{A488}SRC-1 and a doubling of the brightness for the 2:1 species (no change in fluorescence intensity is observed upon binding). The error analysis shows, that as little as 6 % of the putative 2:1 complex would have been detected with a confidence level of 99.7 %. However, although all our models predict significantly higher populations of the 2:1 species than our lower detection limit of 6 %, this species is not detected by the analysis. Therefore, we conclude that only one SRC-1 molecule is present in its complexes with

ERα under all of our experimental conditions. If a complex involving two SRC-1 molecules can be populated under conditions of very high SRC-1 concentration, the affinity for binding the second SRC-1 molecule to the ERα dimer must be significantly lower than that for binding the first (at least a tenfold decrease in affinity).

Finally, we also rule out the possibility that one SRC-1 molecule binds two ERα dimers. If a complex containing two ERα dimers and a single SRC-1 monomer were populated, due to the large difference in molecular weight compared to a complex containing only one ERα dimer, one would expect to observe a much larger change in anisotropy in the equilibrium titrations (see below Figure 5, which involve high ratios of ER HBD/SRC-1, up to 50-fold) as compared to the stoichiometric titrations (Figure 3, in which this ratio did not exceed threefold). However, the total anisotropy change (35 milli-anisotropy units) upon titration of ^{A488}SRC-1 is identical regardless of the total concentration of SRC-1 (and thus the ratio of ER HBD/SRC-1). Thus we conclude that one SRC-1 molecule and one ERα dimer are present in the complex which predominates under all concentration conditions tested.

Modulation of the SRC-1/ER affinity by ligands and response elements

The affinities of ^{A488}SRC-1 for ERHBD, full-length ERα, and full length ERα in the presence of saturating ERE oligonucleotide were determined from anisotropy-based titrations of the ^{A488}SRC-1 (Figure 5(a)-(c)). These binding profiles were fit using a simple model based on the stoichiometry of $(SRC\text{-}1)_1$/ER dimer (Table 2). FCS experiments using ^{A488}SRC-1, ERHBD and ERα at the low concentrations of SRC-1 (5 nM) used in the titrations confirmed this stoichiometry in the presence of all agonists. The affinity of ^{A488}SRC-1 for ERHBD in the presence of E2 is quite high ($K_D = 30$ nM) and is similar for full length ERα, indicating that no additional interaction with this fragment of SRC-1 is conferred by the presence of the AF1 domain. The affinity of the E2-bound ERα for ^{A488}SRC-1 is approximately fourfold lower ($K_D = 116$ nM) in the presence of saturating ERE, indicating that DNA binding introduces mild energetic constraints against SRC-1 binding. We note that at concentrations greater than 5 nM ERα the receptor is fully bound to its ERE, and that the ER-ERE affinity is independent of the state or type of ligation (Boyer *et al.*, 2000).

In all cases, E2 and E3 exhibited similar efficiencies for promoting ER-SRC-1 complex formation (Table 2), whereas E1 was two to threefold less efficient, consistent with the PPDA experiments (Figure 1). It is interesting that the final anisotropy values obtained from the fits of the data in Figure 5(b) and (c) for the ^{A488}SRC-1/ERα complexes were significantly lower in the presence of E1 and E3 compared to that observed in the

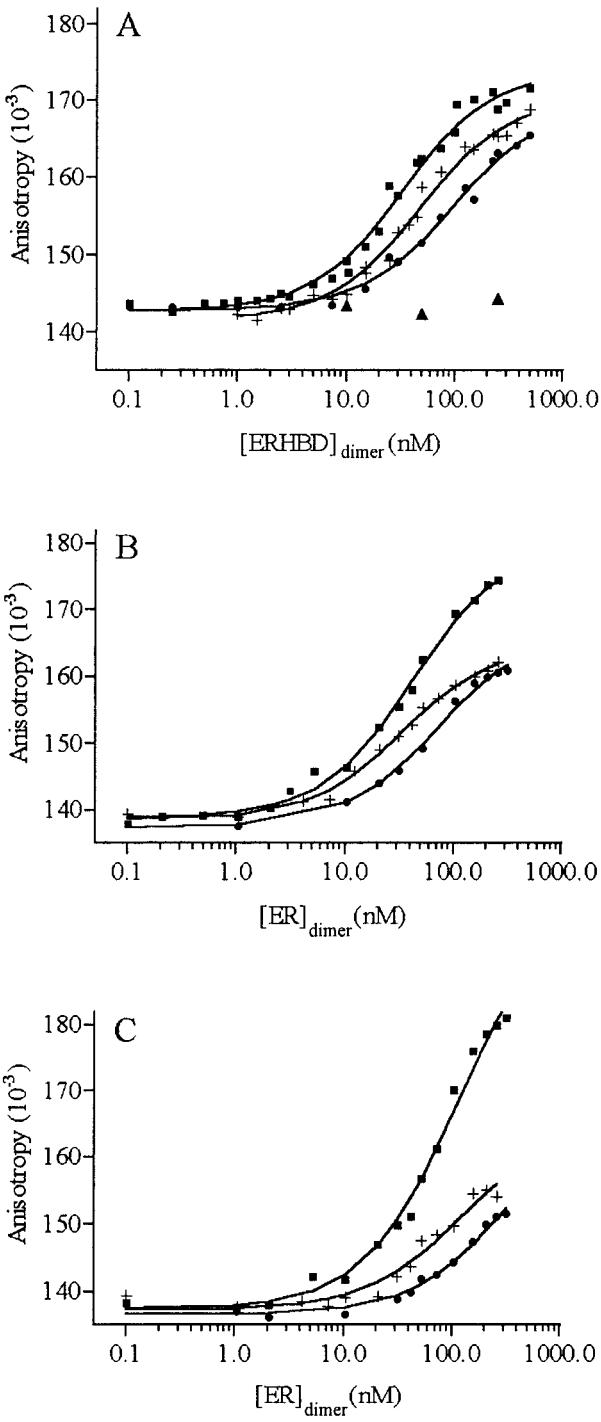

Figure 5. Titration of ^{A488}SRC-1 by ERHBD, ERα and ERα + ERE. Titrations of ^{A488}SRC-1 (5 nM) by (a) ERHBD, (b) ER full-length and (c) ER full-length + ERE, in the absence (▲) or in the presence of ER agonists (5 μM):E1 (○), E2 (□), E3 (+). Receptor concentrations are expressed as dimers. An increase in the anisotropy values, indicating an interaction, occurs only in the presence of agonists. Each point is the mean of two or three independent measurements. The line through the points represents the fit of the data, using a 1/1 simple interaction model (Table 2). The fluorescence lifetime of the Alexa 488 (4 ns for 98% of the emitting species) does not change upon binding.

Table 2. Dissociation constants (nM) of ^{A488}SRC-1 complexes with ER-HBD, ERα, or ERα + ERE, in the presence of E1, E2 or E3

	Dissociation constants (nM)		
	E1	E2	E3
ER HBD	100 ± 24	30 ± 6	47 ± 9
ERα	69 ± 13	37 ± 5	31 ± 5
ERα + ERE	374 ± 196	116 ± 29	141 ± 76

Uncertainties on the recovered dissociation constants were obtained by rigorous confidence limit testing, which involves refitting the curve at each tested value of the parameter.

presence of E2. For example a plateau value of 199 milli-anisotropy units is recovered for the ERα/ERE/SRC-1 complex in the presence of estradiol, whereas with E1 and E3 we obtain values of 167 and 163 milli-anisotropy units. A lower value for the anisotropy indicates that the dynamic properties of the Alexa dye on the N terminus of the bound SRC-1 fragment are distinct for the complexes with different agonists. This phenomena is not observed for complexes of ^{A488}SRC-1 with the ERHBD, indicating that the difference in fluorophore dynamics in the SRC-1 molecule is induced by the presence of either the ERα DNA-binding domain or the AF1.

Discussion

We have used a variety of approaches (most based on fluorescence spectroscopy) to characterize the interaction of a fragment of the steroid receptor coactivator 1 with the full length human estrogen receptor α or its hormone binding domain. Fluorescence correlation experiments, together with classic steady state approaches, have allowed us to demonstrate unambiguously a stoichiometry of one SRC-1 molecule per ERα dimer (full length or isolated HBD) under all our experimental conditions. PCH analysis of the fluorescence correlation data is a powerful tool for the determination of the stoichiometry of molecular complexes under single molecule conditions (Chen *et al.*, 1999a; Muller *et al.*, 2000). Here, we show that this information can be exploited at higher concentrations, which makes it suitable for the investigation of a large number of biomolecular complexes, including those of modest affinity. In fact, the characterization of the stoichiometry of biomolecular complexes can be one of the most difficult tasks in advancing our understanding of these interactions. A number of techniques are available but each presents its own advantages and disadvantages. For example, analytical ultracentrifugation provides very accurate estimations of molecular weight but requires relatively large concentrations (micromolar) and, for equilibrium measurements, several hours. In our case, the difficulty in obtaining large quantities of the proteins involved and their marginal stability (especially in the case of full-length ERα) precluded the use of analytical ultracentrifugation, although for other applications, in particular, complexes of lower affinity for which the material is not a limiting factor, it would be the technique of choice. PCH analysis on the other hand is very rapid, a few minutes, and requires very low concentrations and small volumes of material. In the present case, PCH analysis is also more accurate than measurement of the translational diffusion coefficient by dynamic light scattering, analytical ultracentrifugation, correlation spectroscopy or rotational diffusion by time-resolved fluorescence anisotropy. In fact, the difference in the molecular brightness for a 2:1 SRC-1/ERα complex would be twofold compared to a 1:1 complex, whereas, the change in translational or rotational diffusion coefficients would be marginal given the low molecular mass of the SRC-1 fragment compared to that of the ERα (a 1:1 complex has a molecular mass of 176 kDa, and a 2:1 complex only increases to 202 kDa).

It is noteworthy that binding of a single SRC-1 molecule to a symmetric ERα homodimer introduces an asymmetry in the complex, which may contribute to the proper orientation of the pre-initiation complex. From this point of view, the estrogen receptor system resembles the homodimeric catabolite gene activator protein (CAP) for which activator ligand binding is anti-cooperative, and whose active functional subunit depends on the promoter architecture, thus leading to an alternate interaction mode with the RNA polymerase (Zhou *et al.*, 1994). In the presence of estradiol, the affinity between SRC-1 and the ERHBD dimer is 30 nM, similar to previously reported results based on less direct methods (Gee *et al.*, 1999; Zhou *et al.*, 1998), but significantly lower than the 1 nM K_D obtained using Biacore measurements on SRC-3 and ERα (Suen *et al.*, 1998). We also found that the affinity of SRC-1 for full-length ERα is comparable to that for ERHBD. Therefore, it is unlikely that this fragment of SRC-1 presents additional interactions with the AF-1 domain of ERα. However, the decrease in affinity between SRC-1 and full-length ERα when bound to the ERE indicates that the DNA-binding domain and its interaction with the ERE alter the properties of the SRC-1 interactions with the HBD. Since many natural response elements for ERα are imperfect palindromes, the ER dimer may undergo asymmetric conformational changes, involving a preferential orientation of the coactivator molecule relative to the promoter.

Of particular interest is the observed modulation of the affinity when estrone is used instead of estradiol, suggesting that the relative potency of different agonists could result from their relative ability to promote interaction between ERα and the coactivator proteins. The effects of the three natural estrogens E2, E1 and E3 (which differ in structure at only two positions (C_{16} and C_{17})) on gene expression is not precisely correlated with their relative affinity for ERα (five times lower for E1 and E3 compared to E2) (Anstead *et al*., 1997; Pilat *et al*., 1993). We have observed previously modulation of receptor-coactivator affinity by agonist structure in studies of the ERα/TIF-1α interaction (Thénot *et al*., 1999). Such modulation could reflect changes in the conformation of the receptor through an altered stabilization of helix 12 by E1 and E3. The lower plateau anisotropy values for the ^{A488}SRC-1/ERα complexes in the presence of E1 or E3 relative to that observed in the presence of E2 do not arise from differences in stoichiometry as confirmed by the FCS experiments, but rather are indicative of altered local dynamics of the Alexa probe in these different complexes. This difference in dynamics is only observed for complexes with the full-length ERα. Paige and co-workers (Paige *et al*., 1999) found that ERα and β may undergo distinct conformational changes depending upon the agonist used. Different conformations of the receptor could serve as well to promote a preferential interaction with specific coactivators, as is the case for the vitamin D receptor, which appears to exhibit a selective interaction with SRC-1 *versus* TIF-2, depending upon the agonist used (Takeyama *et al*., 1999).

The crystal structures of NR hormone-binding domains in various ligation states (Darimont *et al*., 1998; Moras & Gronemeyer, 1998; Nolte *et al*., 1998; Shiau *et al*., 1998) suggest that the repositioning of helix 12 upon agonist binding is crucial for the interaction with the coactivators. While antagonists clearly result in an unfavorable conformation of the helix 12 for coactivator binding, certain ligands could promote an intermediate or dynamic positioning of helix 12 leading to less efficient interaction with the coactivators. It is clear that in addition to *in vivo* studies of hormone effects on cell growth and development, a thorough understanding of hormone-dependant transcriptional regulation will require the characterization of the subtle modifications by hormone of the structure, energetics and dynamics of the nuclear receptor/coactivator complexes.

Materials and Methods

E1, E2 and E3 were purchased from Sigma Aldrich (St Quentin, France), 4-hydroxy-tamoxifen (OHTAM) from Besins Iscovesco (Paris, France), and ICI 182780 (ICI) from AstraZeneca (London, UK). Raloxifen (Ral) was a kind gift from J.-C. Nicolas (INSERM U439, Montpellier, France).

Protein expression and purification

His-tagged proteins were over-expressed in *Escherichia coli* and purified by Ni^{2+}-NTA agarose beads under native conditions (Quiagen, Courtaboeuf, France). DNA encoding the nuclear receptor interacting domain (NID, amino acid residues 570 to 780) was excised from pGex2tk-SRC-$1_{570\text{-}780}$ and subcloned into *Bam*HI and *Sma*I digested pQE-30 (Quiagen) to generate the PQE_{30}-SRC-$1_{570\text{-}780}$ plasmid. pET_{15b}-hERα encoded the HBD of hERα (K302-P552).

The expression and purification of the GST-SRC-1 fusion protein was carried out as previously described by Cavailles *et al*. (1995). The concentration of GST-SRC-1 loaded on the Sepharose beads was estimated by gel electrophoresis using ovalbumin and BSA as standards.

ERHBD concentration was determined by UV absorbance at 280 nm ($\varepsilon = 24000\ M^{-1}\ cm^{-1}$). SRC-1 concentration was determined by the Lowry method using BSA as a standard, due to the weak absorption of the polypeptide at 280 nm and interferences with the alexa dye in Bradford analysis. ERHBD activity was checked using [^{3}H]estradiol binding.

Full-length purified baculovirus-expressed ERα was purchased from Panvera corp (Madison, WI). The concentration of active receptor in each preparation was determined by the supplier by tritiated estradiol binding and compared to the concentration of total protein obtained by Bradford analysis. All others preparations were over 90 % pure as assessed by SDS-PAGE.

Oligonucleotides

HPLC purified oligonucleotides were purchased from Genosys (Cambridge, UK). The target sequence referred to here as ERE has the sequence given below for the sense strand: 5′-AGCTTCGAGGAGGTCACA-GTGACCTGGAGCGGATC-3′.

The sense and anti-sense strands were annealed by heating to 85 °C for ten minutes in 10 mM Tris buffer in the presence of 0.1 mM EDTA, 0.1 mM DTT (pH 7.5) and cooling slowly to room temperature. Proteins and oligonucleotides preparations were stored at −80 °C.

Protein labeling

Alexa 488 and Alexa 568 protein labeling kits and Anti-Alexa 488 antibody were purchased from Molecular Probes (Eugene, OR), and labeling was performed as recommended by the supplier. Further purification was performed by anion exchange HPLC to remove the remaining free fluorophore and unlabeled protein. Removal of free dye was verified by thin layer chromatography. The labeling ratio was verified as one fluorophore/SRC-1 molecule by visible absorption and mass spectrometry.

Protein-protein-DNA assay (PPDA)

PPDA binding assays were performed as described by Thénot *et al*. (1999) using ERα expressing vector transfected COS cell extracts.

Fluorescence experiments

The buffer solution for all fluorescence measurements was 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 % (v/v) glycerol (pH 7.5). Binding assays

were performed using a Beacon 2000 polarization instrument regulated at 4 °C, using filters for fluorescein at a ^{A488}SRC-1 concentration of 5 nM. Anisotropy was measured successively until stabilized, and the reported values are the average of five to eight measurements after stabilization.

Fluorescence measurements on ^{A568}SRC-1 were made using photon counting through a 590 nm high-pass filter on an ISS (Champaign, IL) Koala 2 with excitation at 550 nm. Binding data were analyzed using BIOEQS (Royer *et al*., 1990).

For the fluorescence fluctuation experiments, a femtosecond mode locked Titanium-Sapphire laser (Mira 900, Coherent, Palo Alto, CA) set at 780 nm and pumped by an intracavity doubled Nd:YVO_4 laser (Coherent) was used as the two-photon excitation source. The experiments were carried out using a Zeiss Axiovert 135 TV microscope (Thornwood, NY) with a 63× Plan Apochromat oil immersion objective (N.A. = 1.4). The average power at the sample is less than 7 mW. No photobleaching was observed for any of the samples measured under our experimental conditions. Photon counts were detected with an avalanche photodiode (EG&G, SPCM-AG-151), directly connected to a home-built data acquisition card. The photon sampling frequency was set at 20 kHz. The photon counts were analyzed with programs written for PV-WAVE version 6.21 (Visuals Numerics, Houston, TX), described by Muller *et al*. (2000).

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