

Characterization of the interaction between recombinant estrogen receptor alpha and transcriptional coactivator SCR-1 NR-2 binding motif through fluorescence polarization study with Mithras LB 940

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Introduction

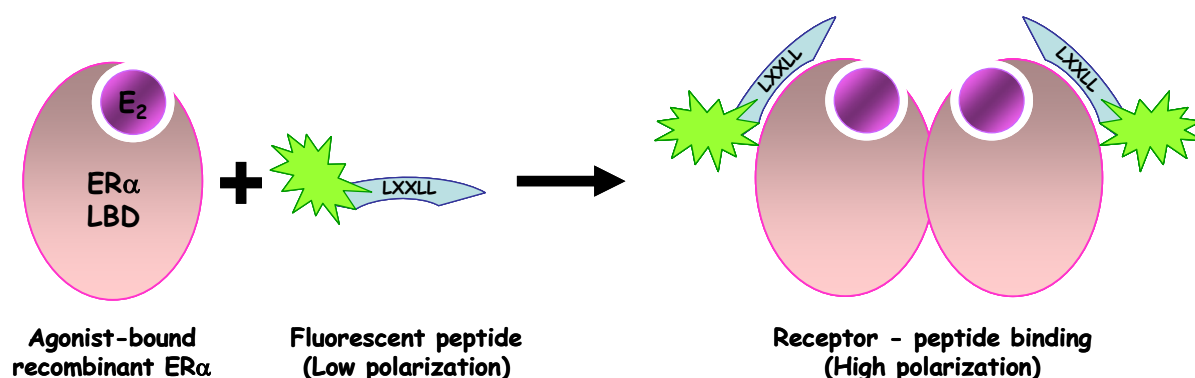


Figure 1: Principle of the recombinant ERα- fluorescein-NR box 2-binding motif of SRC-1 polarization assay. Agonist-bound recombinant ERα adopts a conformation favouring interaction with the fluorescently labelled LXXLL peptide and the resulting larger complex has a higher polarization value.

Hormonal regulation of gene activity is in part mediated by nuclear receptors (NR) acting as ligand-activated transcription factors (Mangelsdorf al., 1995). These proteins interact with the ligand, the DNA-response element and, in an agonist-dependent 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 (AF), AF-1 located in the N-terminal region of the receptor, and AF-2 located in the hormone-binding domain.

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The crystal structures of the ligand-binding domains of several NR (reviewed by Moras and Gronemeyer, 1998) showed the structural role of helix 12 (H12) in the active/inactive switching of 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. The consensus sequence LXXLL called NR-box is the specific motif of coactivators. It is the dominant factor in modulating their binding to 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-1 contains four NR-boxes. Three of these (NR-boxes 1-3) are located near the core protein, and the fourth one, 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 estrogen receptor (ER) ligand binding domain (Mak et al., 1999). ER belongs to the NR superfamily and mediates activation of estrogen-responsive genes. In the current model of ER action, ligand binding induces a conformational change in ER leading to a cascade of events including dissociation of bound repressor proteins, recruitment of coactivator proteins possessing histone acetylase activity, local unwinding of chromatin, and subsequent binding of Pol II and other transcriptional factors. In this study, we quantitatively characterized the interaction of a recombinant ER α produced in bacteria with a fluorescent peptide corresponding to NR box 2-binding motif of the coactivator protein SRC-1 in an *in vitro* high-throughput fluorescence polarization assay using the Mithras LB 940 from BERTHOLD TECHNOLOGIES.

Experimental Procedures

Peptide and ligand

Fluorescent peptide (fluorescein- β A-⁶⁸⁶RHKILHRLQLGS⁶⁹⁸) corresponding to NR box 2-binding motif of SRC-1 was purchased from Neosystem (Strasbourg, France) and was kindly provided by Vivian Pogenberg (CNRS U5048 – INSERM U554, Montpellier, France) and described in Pogenberg et al., 2005. 17 β -estradiol (E₂) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide at 10⁻² M.

Recombinant receptor production

Recombinant ER α ligand binding domain was produced as already described (Pillon et al., 2005). Briefly, hER α LBD(Lys₃₀₂→Pro₅₅₂) Cys_(381,417,530)→Ser triple mutant in fusion with six histidine residue plasmid (Gangloff et al., 2001) was produced in BL₂₁ DE₃ electrocompetent *Escherichia coli* cells (Promega, Charbonnières, France). Recombinant ER α was then purified on Ni-NTA-agarose phase (Qiagen, Courtaboeuf, France). The concentration of active recombinant ER α was measured by a [³H]17 β -estradiol ligand-binding assay.

Fluorescence polarisation assay

Fluorescence polarization assays were performed using the LB 940 Mithras multimode plate reader from BERTHOLD TECHNOLOGIES (Bad Wildbad, Germany) using a 485 nm excitation filter and a 535 nm emission filter.



Figure 2: LB 940 Mithras Multimode reader from BERTHOLD TECHNOLOGIES.

The buffer solution used for the assays was 100 mM Tris-HCl (pH 8), 10% (vol/vol) glycerol and 1 mg/ml BSA. Estradiol was added to the buffer at sufficient concentration (1 μ M) to saturate receptor and fluorescent peptide concentration which was 50 nM each. For sample preparation dilutions were initiated at the highest protein concentration (0.25 nM) and sample was diluted ten times successively, by a factor of 1.8, in 50 nM fluorescent peptide and 1 μ M estradiol containing buffer, at 4°C to avoid recombinant ER α degradation. Then, 100 μ l of each dilution were transferred in a black 96-well plate

(Fluotrac 200, Greiner Bio-One, Germany), to allow us to establish the titration curve. Fluorescence polarization was measured for 1 second per well when the plate temperature was stabilized at about 20°C within the LB 940 Mithras. Halogen lamp energy for fluorescence excitation was fixed at 65,000.

The G-factor (Gf) used in polarization calculation was first determined as being 0.66 for our fluorescein peptide. The experiments were performed both with and without background correction. Background signal (bkg) was obtained with the fluorescent peptide containing buffer without receptor. The results were more in a positive range without background correction due to a shift of all polarisation values.

If the interaction between the two proteins is high, it will be better to use background correction. In any cases the G-factor must be recalculated with or without background correction.

Two emission measurements were needed to evaluate polarization. The first one using a polarized emission filter parallel to the excitation filter (called S-plane) and the second one with a polarized emission filter perpendicular to the excitation filter (called P-plane). Results were expressed in millipolarization (mP) as a function of recombinant ERα amount and $mP = 1000 \times (S-bkg) - [Gf \times (P-bkg)] / (S-bkg) + [Gf \times (P-bkg)]$. Binding affinity of coactivator peptide for recombinant ERα was evaluated by fitting the curve using linear transformation and Scatchard analysis.

Instrument settings

Mithras LB 940 is operated through the Windows® PC software MikroWin 2000 from BERTHOLD TECHNOLOGIES which also served as a data evaluation tool. The standard configuration pre-set parameter files for Fluorescence Polarisation readings and G-factor determination are supplied which may be modified according to individual demands.

First the G-factor of the selected protein has to be determined.

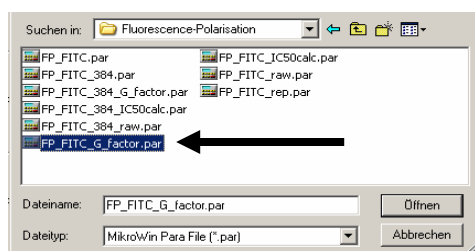


Figure 3: Selection of G-factor determination parameter file.

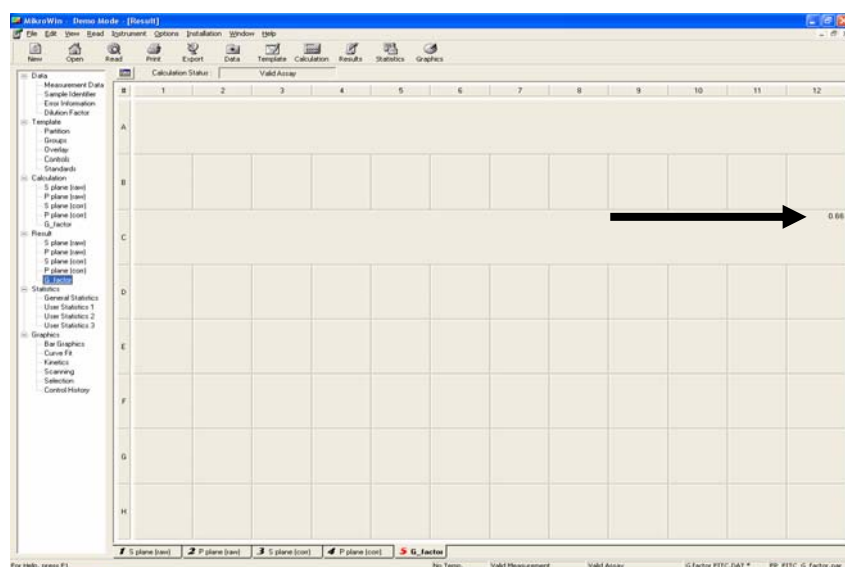


Figure 4: Result window: the matrix showing the calculated G-factor.

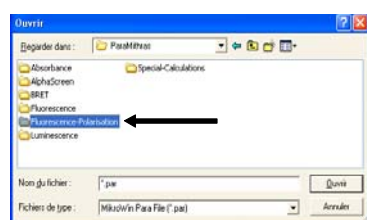


Figure 5: Selection of the Fluorescence Polarisation parameter file.

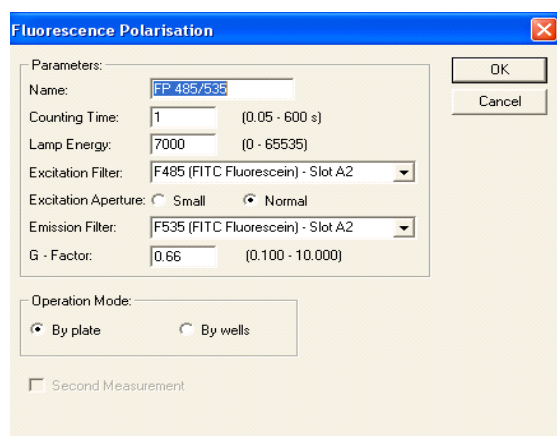


Figure 6: Fluorescence Polarisation operation dialogue.

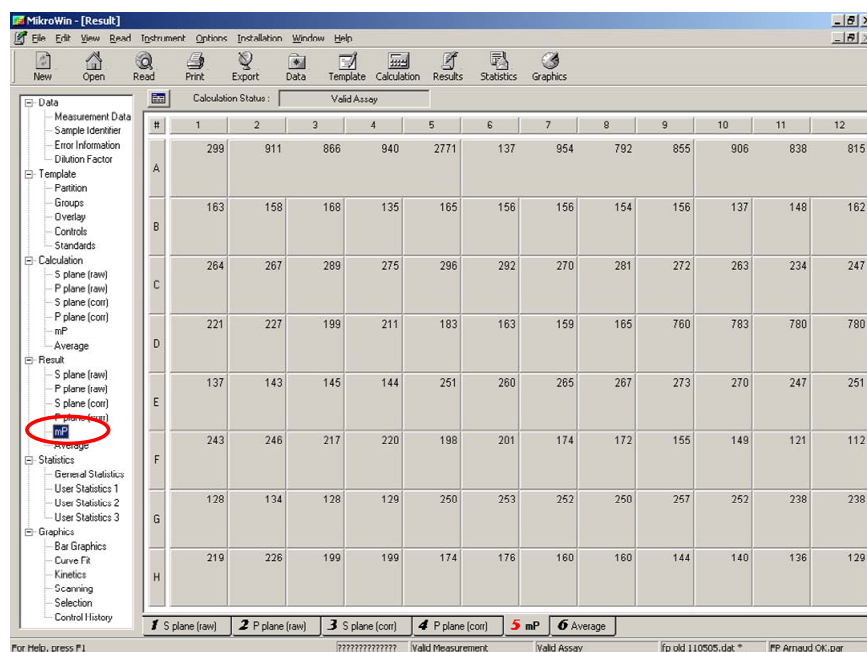


Figure 7: Result window: Matrix showing the calculated mP values.

Results

In order to obtain large amounts of ER α , we decided to produce a mutant ER α ligand binding domain which would be highly expressed in bacteria. Three of its cysteine residues (381, 417, and 530) were mutated into serine residues, which circumvented aggregation and denaturation problems. The mutant protein bound estradiol with wild type affinity but had limited transcriptional capacity (Gangloff et al., 2001). Furthermore, recombinant ER α was purified on a Ni-NTA-agarose phase in order to clean receptor from bacteria proteins (Pillon et al., 2005) and concentration of active recombinant ER α was measured by a [3 H]17 α -estradiol ligand-binding assay that gave a 10 μ M purified recombinant ER α solution.

The fluorescent peptide is composed of a fluorescein- β A probe covalently coupled to the N terminus of the NR box 2-binding motif of SRC-1 686 RHKILHRLQLQGS 698 . This peptide contains the LXXLL motif required for ER (Pogenberg et al., 2005).

preliminary experiments, we determined the peptide amount sufficient to perform fluorescence polarization assays. Fluorescence detection was thus achieved with successive peptide dilutions to find out the LB 940 Mithras limit of sensibility and efficient fluorescent peptide concentrations (Fig.8).

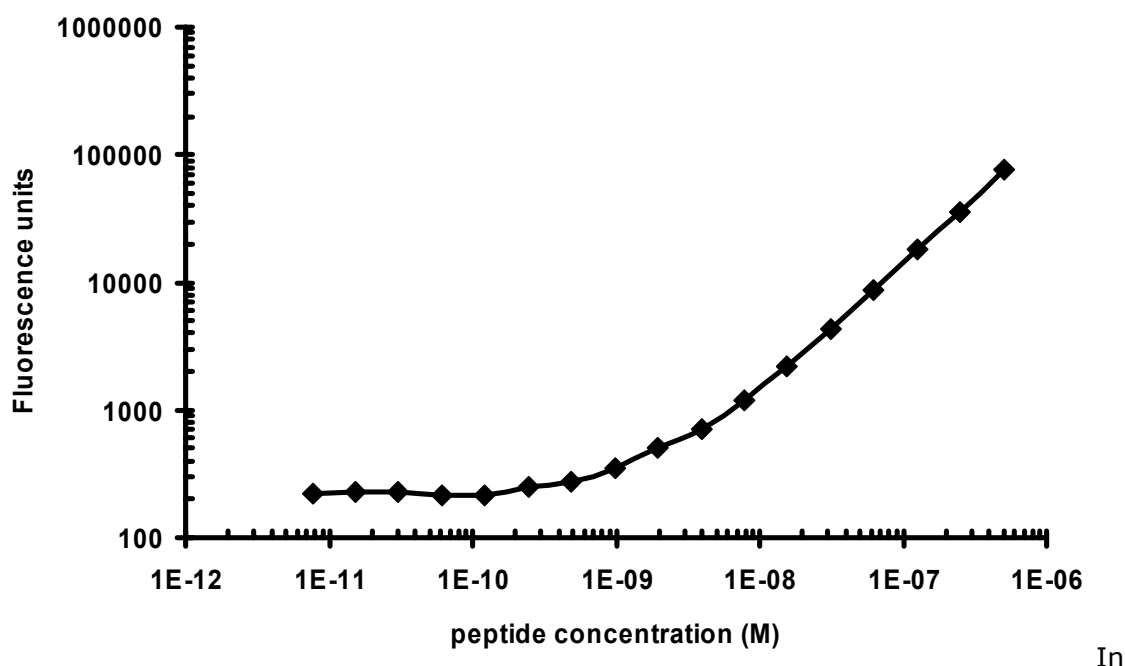


Figure 8: Fluorescence assay of dilution series of fluorescein-NR box 2-binding motif of SRC-1. Peptide dilution series were prepared and transferred in 96-wells that were introduced into the LB 940 Mithras. The LB 940 Mithras was able to detect 1 nM peptide but the response was perfectly linear from 5 nM on.

The Mithras was able to detect 1 nM fluorescent peptide but a linear response was obtained at about 5 nM. We thus decided to use a ten fold higher concentration and a 50 nM efficient SRC-1 fluorescent peptide concentration was then chosen for fluorescence polarization assays.

The fluorescent peptide interaction with recombinant ER α ligand binding domain was monitored by measuring changes in the steady state fluorescence polarization (Fig.9).

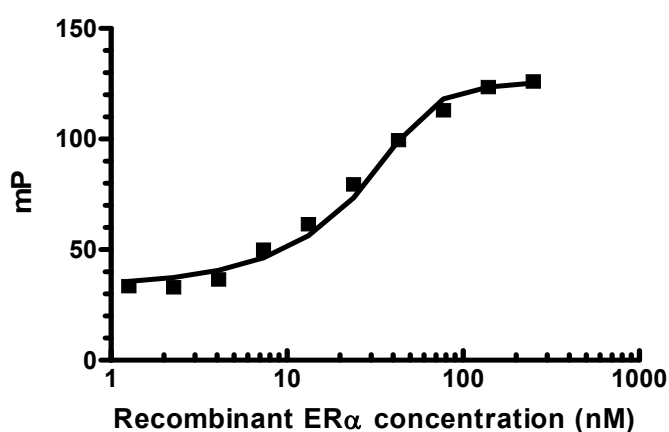


Figure 9: Titration of fluorescein-NR box 2-binding motif of SRC-1 by recombinant ER α in polarization assay. Peptide and dilution series of recombinant ER α in presence of estradiol were prepared and transferred in 96-wells that were introduced into the LB 940 Mithras. Higher polarization values (mP) correspond to greater interaction between recombinant ER α and fluorescent peptide. Each point is a mean of three independent measurements. Fluorescent coactivator peptide binding affinity for recombinant ER α was determined in Figure 10.

The polarization value is the intensity normalized difference between parallel and perpendicular polarized fluorescence emissions and is inversely proportional to the rotational diffusion, thus the larger the molecule, the higher the polarization (Lakowicz, 1991). Dilution series of recombinant ER α in presence of estradiol were achieved in 50 nM peptide containing buffer and the equilibrium was established rapidly. Each sample (100 μ l) was then transferred in a black 96-well plate and introduced into the Mithras LB 940 for fluorescence polarization detection (Fig.9). Binding affinity of coactivator peptide for recombinant ER α in presence of estradiol was evaluated by fitting the curve using a linear transformation (Fig.10). Scatchard analysis allowed us to determine a K_d value of 7.5 nM. This dissociation constant was in accordance with those found in the literature using fluorescence polarization but with different instruments (Margeat et al., 2001; Ozers et al., 2005).

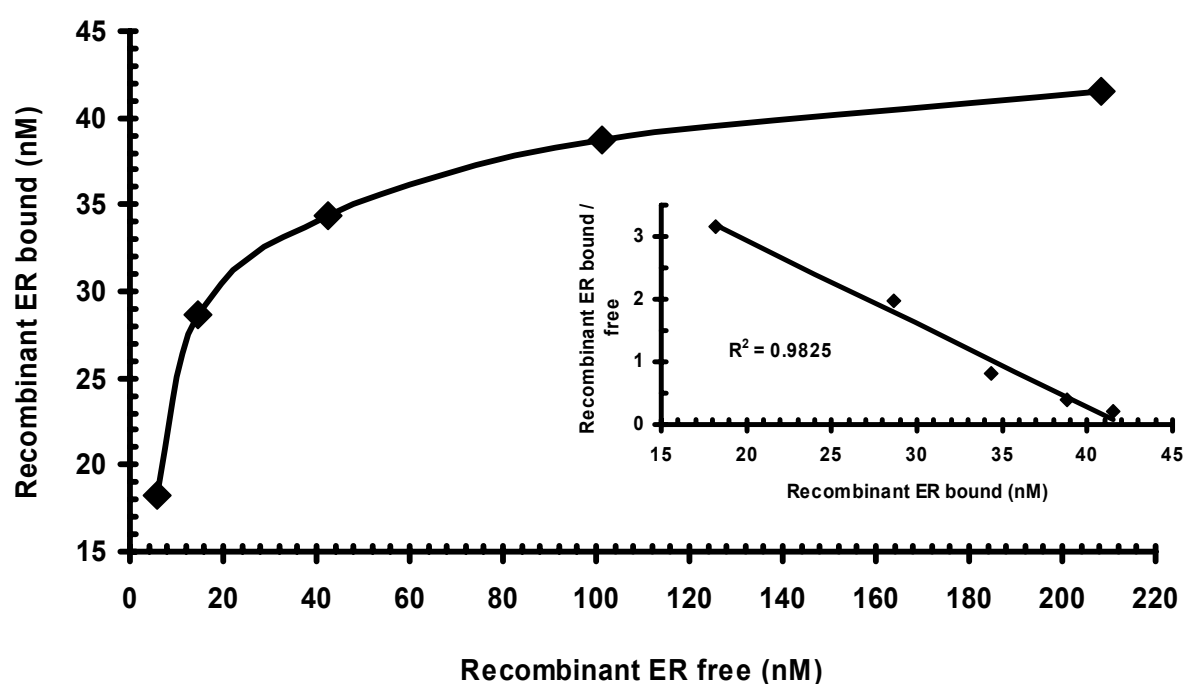


Figure 10: Fluorescein-NR box 2-binding motif of SRC1 binding ability of recombinant ER α . Binding of SRC-1 peptide to recombinant ER α was performed in presence of estradiol. Fluorescence polarization values represented in Fig.3 were used to determined bound and unbound peptide concentration. Specific bound concentration was calculated after non-specific bound counts were subtracted from total bound counts. (Inset) Linear Scatchard transformation of specific binding gave a K_d of 7.5 nM for recombinant ER α .

Conclusion

We used an approach based on fluorescence polarization to characterize the interaction of the NR box 2-binding motif of SRC-1 coactivator with a recombinant ER α ligand binding domain. In presence of estradiol, the affinity between those two proteins was 7.5 nM. The affinity value we obtained was within the same order of magnitude as previously reported values that were based on other methods (Gee et al., 1999; Zhou et al., 1998, Cheskis et al., 2003). LB 940 Mithras was very convenient to perform fluorescence polarization in multi-well plates with such a stable receptor exhibiting high affinity for coactivators. As the dissociation constant is extremely temperature dependent, experiments were to be performed at as low a temperature as possible. This assay highlighted the usefulness of fluorescent LXXLL-peptide as a tool to probe ligand-dependent recruitment of coactivator to receptors. Working in multi-plates was easier than in tubing system and experiments were performed very fast (less than 2 minutes to read one plate and to get results).

Material list

- 17 β -Estradiol (Sigma chemical Co., St. Louis, MO, USA)
- Escherichia coli cell (Promega, Charbonnières, France)
- Ni-NTA Agarose phase (Qiagen, Cortaboef, France)
- Mithras LB 940 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany)
- Buffer (100 mM Tris-HCl (ph 8), 10 % (vol/vol) glycerol, 1 mg/ml BSA)
- Microplates 96 well, black (Fluotrac 200, Greiner Bio-One, Germany)

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