Development of a time-resolved Förster resonance energy transfer (TR-FRET) coactivator displacement assay for ERRα and ERRγ

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Introduction

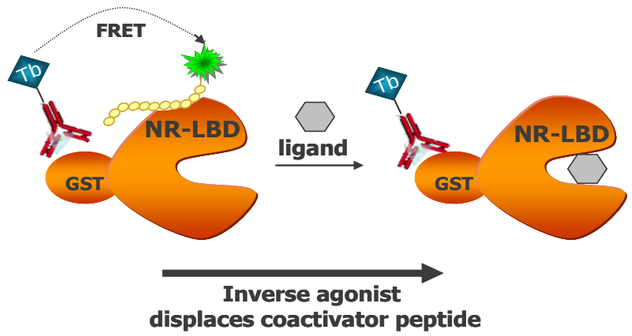
Estrogen-related-receptors (ERRs) are constitutively-active orphan nuclear receptors (NRs) that are closely related to estrogen receptors and have been shown to be potential biomarkers and targets for treatment of breast cancer and obesity. Although no natural ligand is known, ERRα is deactivated by XCT790 and ERRγ is deactivated by estrogenic compounds such as diethylstilbestrol (DES), tamoxifen, and 4-hydroxytamoxifen (4-OHT). To assess how peptides derived from coregulator proteins interact with ERRα and ERRγ in response to ligand, an *in vitro* interaction methodology using time-resolved Förster resonance energy transfer (TR-FRET) was developed using GST-tagged ERRα and ERRγ ligand binding domains (LBD), a terbium-labeled anti-GST antibody, fluorescein-labeled peptides containing sequences derived from coregulator proteins, and various ligands. An initial screen of these coregulator peptides bearing the coactivator LXXLL motif, the corepressor LXXI/HIXXXI/L motif or other interaction motifs from natural coactivator sequences indicated that peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) interacted strongly with both NRs in absence of ligand. Fluorescein-labeled PGC1α was displaced from the ERRα or ERRγ LBD in the presence of increasing concentration of XCT790 or 4-OHT respectively, but DES and tamoxifen were less effective in PGC1α displacement. These findings also suggest that binding of different ligands to ERRα and ERRγ results in differential affinity of coregulators for ERRα/ERRγ due to unique ligand-induced conformations. In summary, our results describe a high throughput methodology to examine ERR-coactivator interactions in a TR-FRET-based assay that may be used in the discovery of novel drugs for the treatment of breast cancer.

Methods

Materials - GST-tagged ERRα and ERRγ, Tb-labeled anti-GST antibody, fluorescein labeled peptides and assay buffers were from Invitrogen (Madison, WI). Ligands and the LOPAC^{1280} library were from Sigma-Aldrich (St. Louis, MO). All assays were conducted in low volume black 384-well plates from Corning (Acton, MA) using a total volume of 20 µL.

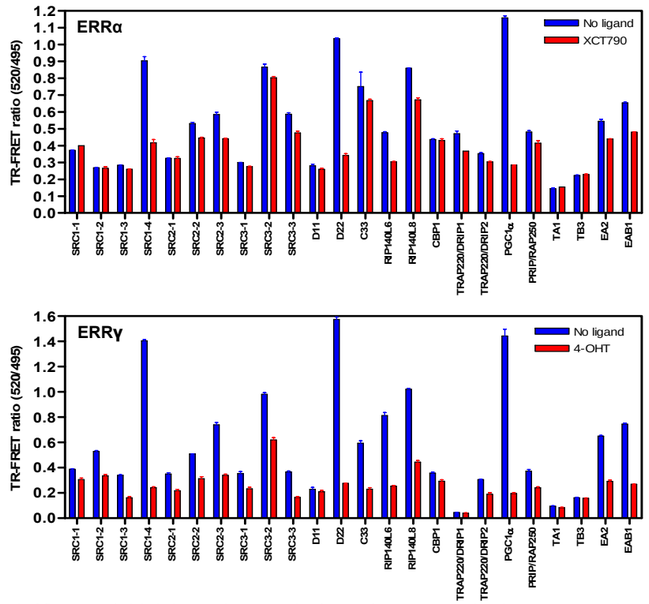
ERR Coactivator Displacement Assay – All assays were performed using 5 nM Tb-anti-GST, 500 nM PGC1α-Fl, and either 10 nM ERRα or 5 nM ERRγ (unless otherwise indicated). Incubations were conducted at room temperature at indicated time points prior to measurement of TR-FRET using a Tecan Ultra instrument (Durham, NC) with excitation at 340 nm (30 nm bandwidth) and emission at 495 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth), 100 µs lag time, 200 µs integration time and data were fit using XLfit4 or GraphPad™ Prism®.

Figure 1 – Principle of Coactivator Displacement Assay



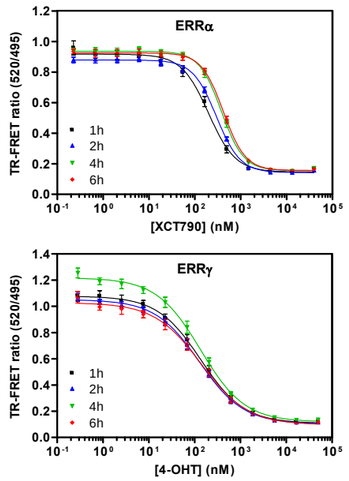
Unliganded ERRα and ERRγ are in a constitutively active conformation, leading to association with specific LXXLL-bearing coactivator derived peptides. This association is detected by FRET from the Tb-labeled antibody to a fluorescein-labeled coactivator peptide. Binding of inverse agonist to the NR causes a conformational change that results in a decrease in the affinity of the NR for the coactivator peptide and a loss of TR-FRET signal.

Figure 2 – Coregulator peptide screen



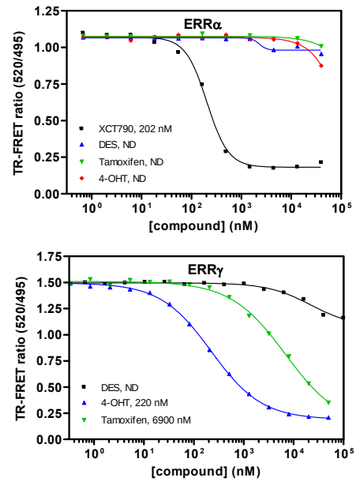
A panel of fluorescently labeled coregulator peptides (500 nM) were screened in the presence or absence of 10 µM XCT790 (ERRα) or 4-OHT (ERRγ). The constitutive association of ERR with a given coactivator peptide is indicated by higher TR-FRET ratios in the absence of ligand (black), and disruption of the peptide-receptor interaction by the indicated ligand is indicated by a decrease in TR-FRET ratio (red).

Figure 3 – ERR Coactivator Assay Stability



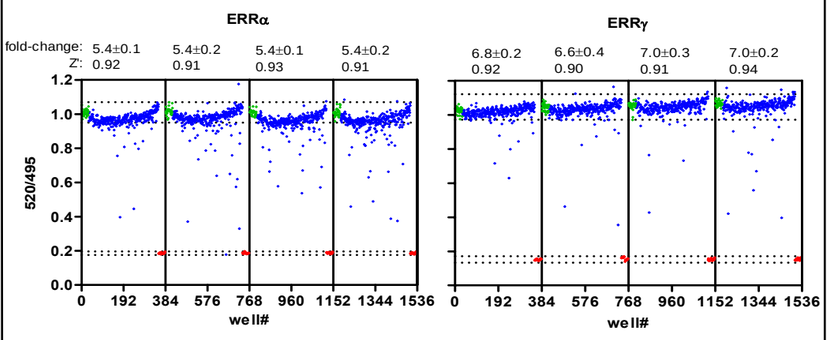
Increasing concentrations of ligand were assayed using the conditions outlined in the methods section and TR-FRET was measured at varying timepoints.

Figure 4 – Effect of ligands on Fl-PGC1α displacement



Serial dilutions of selected ligands were assayed using the conditions outlined in the Methods section and a 2 hour incubation time.

Figure 5 – LOPAC^{1280} Screen



The Library of Pharmaceutically Active Compounds (LOPAC^{1280}, Sigma-Aldrich) was screened at 10 µM (1% DMSO) to demonstrate the application of the TR-FRET coactivator displacement assay to identify ligands that disrupt the association between ERRs and PGC1α. The screen was automated using a Biomek FX (Beckman-Coulter) for all compound handling and assay reagent dispensing. Dotted lines represent ±1 standard deviation of control wells averaged over the entire screen. TR-FRET of each library compound is indicated in blue, wells containing no ligand are indicated in green, and 10 µM XCT790 or 4-OHT are indicated in red. The fold-change and Z'-factor values are indicated above the corresponding assay plate results.

Table 1 – LOPAC^{1280} Screen Summary

	ERRα	ERRγ
# compounds screened	1280	1280
total hits	12	6
confirmed hits	6	2
ligands identified	PPT CDTCD MK-886 L-655,240 TTNPB aurintricarboxylic acid	PPT tamoxifen

Table 2 lists the summary of hits generated in the LOPAC^{1280} screen that displayed ≥50% peptide displacement at 10 µM and those that were confirmed by follow-up dose-response studies. PPT: 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole, CDTCD: (R,R)-*cis*-diethyltetrahydro-2,8-chrysenediol, TTNPB: (E)-4-[2-(5,6,7,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benjoic acid.

Results and Conclusions

- Our results describe a methodology to examine interaction of coregulator peptides with ERRα and ERRγ, for use in the discovery of potential drug targets for breast cancer and obesity.
- This approach can be used to screen a panel of coregulator peptides to look for different peptide "fingerprints" of various ligands, indicating different conformational changes. In our results, coactivator peptide PGC1α interacted most strongly with both ERRα and ERRγ in the absence of ligand followed by D22 and SRC1-4.
- The statistical parameter Z' factor that measures the robustness of the assay was greater than 0.8 for displacement of PGC1α from ERRα/ERRγ LBD in the presence of ligand over an assay incubation time of 1-6 hr, indicating an excellent assay.
- Using PGC1α as the coactivator peptide, various ligands showed expected rank order potency when analyzed in a dose response for ERRα and ERRγ .
- The LOPAC^{1280} screening results demonstrate that the ERR assays are amenable to automation and are robust in a high-throughput format. In addition to the known ERRγ ligand tamoxifen, a number of novel ligands for ERRα/ERRγ were identified (Table 2) by the screen.