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An estrogen receptor chimera senses ligands by nuclear translocation

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Abstract

We have developed a new mammalian cell-based assay to screen for ligands of the estrogen receptor. A fluorescently tagged chimera between the glucocorticoid and the estrogen receptors, unlike the constitutively nuclear estrogen receptor, is cytoplasmic in the absence of hormone and translocates to the nucleus in response to estradiol. The chimera maintains specificity for estrogen receptor α ligands and does not show cross-reactivity with other steroids, providing a clean system for drug discovery. Natural and synthetic estrogen receptor α agonists as well as phytoestrogens effectively translocate the receptor to the nucleus in a dose-dependent manner. Antagonists of the estrogen receptor can also transmit the structural signals that result in receptor nuclear translocation. The potency and efficacy of high-affinity ligands can be evaluated in our system by measuring the nuclear translocation of the fluorescently labeled receptor in response to increasing ligand concentrations. The chimera is transcriptionally competent on transient and replicating templates, and is inhibited by estrogen receptor antagonists. Interestingly, the nucleoplasmic mobility of the chimera, determined by FRAP analysis, is faster than that of the wild type estrogen receptor, and the chimera is resistant to ICI immobilization. The translocation properties of this chimera can be utilized in high content screens for novel estrogen receptor modulators.

Keywords: Chimera; Nuclear translocation; Estrogen; Glucocorticoid; Drug discovery; Cell-based assay; High content screen; Mobility

1. Introduction

The estrogen receptor (ER), a member of the steroid receptor family, mediates the primary biological effects of estrogens. Treatment of certain conditions such as breast and ovarian cancers, osteoporosis, and menopause rely on estrogen receptor ligands to either inhibit or reactivate the actions of the estrogen receptor. Because of their medical significance, the search for novel estrogen receptor ligands and selective receptor modulators has been a priority in both endocrinology and medical pharmacology. A number of groups have designed effective systems to monitor the presence of estrogenic activities in environmental and food samples [1–4]. There is a need for accurate methods to screen for new compounds that could modulate receptor activity in mammalian cells and thus serve therapeutic purposes. The most commonly used screening assays currently measure the proliferative effects of estrogens, or the transcriptional activity of the ER or evaluate in vitro or in yeast the estrogenic activity of unknown compounds [5–9].

We and others have previously shown that the glucocorticoid receptor (GR) fused to the green fluorescent protein

Abbreviations: ER, estrogen receptor; GR, glucocorticoid receptor; GFP, green fluorescent protein; Tet, tetracycline; LBD, ligand binding domain; Dex, dexamethasone; E2, 17-β-estradiol; PPT, 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol; DES, diethylstilbestrol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; HSP90, heat shock protein 90; MMTV, mouse mammary tumor virus long terminal repeat; SERM, selective estrogen receptor modulator; YES, yeast estrogen screen

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(GFP) is found predominantly in the cytoplasm, and translocates rapidly to the nucleus in response to ligand [10,11]. The movement of the GR from one cellular compartment to another can be monitored by fluorescence microscopy and provides a tool for drug discovery. Like other nuclear receptors, the estrogen receptor shuttles between the cytoplasm and the nucleus but its constitutive location is predominantly nuclear [12–16], so it does not undergo translocation to the nucleus in response to ligand. Our earlier studies showed that the translocation properties of GR can be conferred to the ligand binding domain of another nuclear receptor, the retinoic acid receptor [17]. We therefore initiated efforts to generalize the concept that the intrinsic translocation properties of GR can be transferred to non-translocating receptors.

In this report, we describe the construction and characterization of a fluorescent glucocorticoid–estrogen receptor chimera that localizes to the cytoplasm in the absence of estrogens but translocates to the nucleus in response to all ERα ligands tested, while remaining unresponsive to glucocorticoids. The efficacy and potency of high-affinity ligands can be measured in our system through dose response analysis of translocation dynamics. Because the translocation of the chimera is monitored by the accumulation of fluorescence in the nucleus, the assay can be automated and adapted for high throughput screening applications. Furthermore, since the chimera is transcriptionally active on both transient and integrated templates, secondary functional assays can be performed on candidate ligands to determine their agonistic/antagonistic activity. Finally, the chimera described here can serve as a new tool in the study of nuclear receptor structure and function, particularly in elucidating the process of nuclear translocation and receptor mobility.

2. Materials and methods

2.1. Automated fluorescence measurements

3617 cells were plated at 2500 cells/well on an optical bottom 96-well plate in the absence of tetracycline to induce the expression of GFP–GR. The following day, cells were washed four times and fresh DMEM supplemented with 5% charcoal stripped serum was added. Twenty-four hours later, cells were washed and the media was replaced with PBS supplemented with 5% charcoal stripped serum and 2 μg/ml Hoechst 33342 stain. After 2 h, cells were treated with 0, 1, 10, or 100 nM dexamethasone and incubated for 15 min before scanning. Live cell scanning was carried out on a Q3DM platform composed of an inverted Nikon TE300 fluorescence microscope (equipped with a robotic stage, a CCD camera and LED phase contrast light source), a mercury arc lamp, fiber optic housing and a control unit. When using live cells, penetration of the Hoechst dye is incomplete and therefore the nuclear mask used to define the nucleus is not visible in all cells. Fluorescence in the GFP channel overlapping the areas of Hoechst fluorescence in the blue channel (pseudo-colored red in Fig. 1) defines the nuclear fluorescence. Algorithms built into the Q3DM system are used to define the full area occupied by each cell based on region expansion outward from the nucleus and cell boundary determinations. The percent nuclear over total fluorescence for each cell is then calculated. Cell line 3134 [18] containing the integrated GFP-tagged GRER construct were plated on Nunc 96-well glass bottom plates and grown overnight. Cells were treated with increasing concentrations of estradiol for 4 h, then washed in PBS, fixed with 4% formaldehyde and stained with 2 μg/ml Hoechst 33342 for 1 h in media containing DMSO or estradiol. Nuclear versus total cell fluorescence was measured on a Discovery-1 system (Molecular Devices, Downingtown, PA). Images were captured using a 20X Plan Fluor objective at four sites per well. GFP expression was visualized using a FITC filter (470/535) with a 400 ms exposure. Nuclei were visualized using a DAPI filter (405/465) with a 30 ms exposure. Images were analyzed using a protein translocation journal from Molecular Devices. The nuclei are defined by the Hoechst stain, and the cytoplasm is measured by region expansion from the nucleus. Correlation coefficients were used for analysis of the nuclear translocation. Correlation coefficient values compare the overlap of the GFP and Hoechst signal, which have a theoretical range between −1 and +1.

2.2. Construction of chimeric receptors

pCI-nGFP-C656G containing a polylinker replacing sites downstream of the coding region of helix 1 of the LBD of rGR [17] was digested sequentially with *Stu*I and *Eco*RI at the polylinker. The ERα expression vector HEGO was digested with *Blp*I and the overhang filled in prior to digestion with *Eco*RI. The *Blp*I–*Eco*RI fragment of HEGO corresponding to partial ERα LBD sequences was inserted into the *Stu*I–*Eco*RI digested vector downstream of partial GR coding sequences. The final construct is shown in Fig. 2 and results in a partial duplication of the loop between helices 1 and 3 of the receptor. GRERΔloop was similarly made using a *Hin*dIII–*Eco*RI fragment of hER, which when inserted into the *Stu*I–*Eco*RI digested vector results in a partial deletion of the loop between helices 1 and 3 of the LBD.

2.3. Cell culture, transient transfections and development of stable cell lines

Cells were maintained in DMEM media supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 1 mM sodium pyruvate. For 3617 cells, the media was supplemented with 5 μg/ml tetracycline to inhibit expression of GFP–GR, unless otherwise stated. Transient transfections of all cells were performed following the manufacturer's protocol using either Fugene 6 or Lipofectamine 2000 reagents. In the case of C127 derived cell lines, cells were transiently transfected