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Investigation of the functional properties and subcellular localization of alpha human and rainbow trout estrogen receptors within a unique yeast cellular context

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ABSTRACT

Estrogens are steroid hormones that play a pivotal role in growth, differentiation and function of reproductive and non-reproductive tissues, mediated through estrogen receptors (ERs). Estrogens are involved in different genomic and non-genomic cell signaling pathways which involve well-defined subcellular ER localizations. Thus, ER activity results from complex interplays between intrinsic binding properties and specific subcellular localization. Since these two factors are deeply intricate, we carried out, in a unique yeast cell context, a comparative study to better understand structure/function/subcellular distribution relationships. This was carried out by comparing two ERs: the human ER α subtype (hERα) and the short form of the α isoform of the rainbow trout ER (rtERα$_S$). Their distinct binding properties to agonist and antagonist ligands and subcellular localizations were characterized in *Saccharomyces cerevisiae* yeast cells. An unexpected partial agonistic effect of ICI 182-780 was observed for rtERα$_S$. Concomitant to distinct binding properties, distinct subcellular localizations were observed before and after ligand stimulation. Due to the unique cell context, the link between ERs intrinsic binding properties and subcellular localizations is partly unveiled and issues are hypothesized based on the role of cytoplasmic transient complexes which play a role in the ER cytoplasmic/nuclear partition, which in turn is critical for the recruitment of co-regulators in the nucleus.

1. Introduction

Estrogens are steroid hormones that play an important role in growth, differentiation and function of reproductive and non-reproductive tissues. Most of their actions are mediated through estrogen receptors (ERs) belonging to the superfamily of the nuclear receptors. Upon hormone binding, ER regulates the transcription of specific, cell type-dependent, genes.

The estrogen receptor, widely expressed in eukaryotes, is mainly present as two subtypes, namely ERα and ERβ. These two ER subtypes, encoded by two distinct genes and expressed in many tissues, exhibit distinct tissue-specific expression levels and distinct functional and biological properties [1–6]. For each subtype, various isoforms may be generated from either alternative exon splicing, or presence of several differentially activated promoters on a same gene, or genome duplication. In human, at least four α isoforms and five β isoforms of ER have been identified [7–10]. In rainbow trout (*Oncorhynchus mykiss*), two isoforms for each ER subtype have been identified, namely rtERα1, rtERα2, rtERβ$_1$ and rtERβ$_2$ [11–14]. Intracellular ERs, in rainbow trout, regulate the vitellogenin synthesis [15].

Abbreviations: E2, 17β-estradiol; E$_S$, estrogens; ER, estrogen receptor; ERE, estrogen response element; LBD, ligand-binding domain; DBD, DNA-binding domain; ERE$_{cs}$, consensus estrogen response element; hERα, human estrogen receptor alpha subtype; rtERα$_S$, short isoform of alpha subtype of rainbow trout estrogen receptor; MCF-7, human breast adenocarcinoma cell line; AF-1 and AF-2, trans-activation functions 1 and 2; RAL, raloxifene; DES, diethylstilbestrol; 4-OHT, 4-hydroxytamoxifen; ICI, ICI 182-780; *rtvtg*ERE, ERE found in the intergenic region of the rainbow trout vitellogenin gene; GFP, green fluorescent protein; ONPG, ortho-nitrophenylgalactoside; EC50, effective concentration for half maximal response; SHM, syrian hamster myometrium; COS-1, monkey kidney fibroblast cell line.

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All ERs share a common structural organization with 6 structural domains (designated as A–F domains), more or less conserved among all ERs from various species and which exhibit different functions [8]. Two distinct transcriptional activation functions (AFs) are located in the NH_2-terminal A/B region and the COOH-terminal E region (named AF1 and AF-2, respectively) of ER. AF-1 is responsible for the ligand-independent transactivation, whereas the AF-2 activity depends on ligand binding. AF-1 activity depends on the phosphorylation state of Ser118 residue of human ERα [16]. Additionally, AFs activity is modulated by the recruitment of coregulators [17,18]. Both AFs may act independently or in a synergistic manner, leading to maximal ER transcriptional activity [19]. The C domain, also called DNA-binding domain (DBD), the highest conserved ER domain, is composed of 2 zinc fingers followed by a sequence rich in basic amino-acid residues. This domain specifically interacts with cognate DNA sequences, called estrogen responsive elements (ERE); these motifs can be the consensus palindromic ERE sequence, AGGTCAnnnTGACCT, but also imperfect EREs or ERE half-sites. The E domain not only contains AF-2 but also the ligand-binding domain (LBD) and a strong dimerization interface. Finally, the D domain is a flexible linker peptide between the DBD and the LBD, and the F domain is considered as a carboxy-terminal extension of the LBD.

It is well established that ER acts as an estrogen-activated transcriptional factor and modulates gene expression by directly binding to ERE DNA sequences located in target genes promoters or by interacting with other transcriptional factors. The classical model of estrogen action proposes that upon estrogen binding, receptors are released from inactive transient complexes containing heat-shock proteins and immunophilins, dimerize and can thereafter bind specific DNA ERE motifs [20,21]. ERE-bound ERs then recruit a series of coactivators such as the p160 coactivators and CREB-binding protein [18]. These ER-coactivators complexes may modulate the chromatin structure and facilitate access of the transcriptional machinery to DNA. Other mechanisms of estrogen action have been proposed in which ERs-regulated gene transcription is due to protein–protein interactions with other classes of transcription factors, and/or with components of other signaling cascades. Control of gene transcription requires a nuclear localization of ER. Thus, the import of ER into the nucleus is an additional way of gene expression regulation.

In this study, were compared the functional features of two estrogen receptors, hERα and rtERα$_S$. hERα (NP_000116.2; 595 amino acid residues) and rtERα$_S$ (CAB45140.1; 577 amino acid residues) exhibit a low sequence homology (41.6%). The DBD and LBD are the most conserved domains with 92% and 60% homology, respectively. Both specifically bind estradiol (E2) with a high affinity, although rtERα$_S$ exhibits a 10-fold reduced estradiol affinity as compared to hERα [22]. Both also stimulate the transcription of genes containing an ERE in response to E2 binding, whereas only rtERα$_S$ can activate these genes in a ligand-independent manner [23].

Activation of ER is therefore complex in nature and highly regulated in a tissue specific manner. As a matter of example, ER pharmacology has to consider concomitant curative effects of anti-tumoral drug (e.g., tamoxifen) and deleterious effects on off-target tissues or organs. We pose that, from the ER binding pattern, i.e., the set of thermodynamic and kinetic binding properties with agonist and antagonist partners, derives particular patterns of subcellular trapping within either transient complexes or specific or non-specific DNA targets in association with recruited co-regulators. Due to a high level of intricacy, it is extremely difficult to identify which property derives from the ER intrinsic properties and which derives from a particular cell context. This is a major pitfall to design new anti-tumoral drugs. Thus, to improve our understanding of the mechanisms involved in ER signaling pathways, we compare, within a unique cellular context, i.e., *Saccharomyces cerevisiae,* and a unique ERE, the binding and functional properties, namely cellular activity and subcellular localization, of the hERα and rtERα$_S$ receptors, in response to agonists, estradiol and diethylstilbestrol, and antagonists, 4-OH tamoxifen, raloxifen (2 known partial antagonists in human cells) and ICI 182-780 (pure antagonist in human cells). This cellular system is widely used for expressing recombinant ERs and quantifying their ability to enhance gene transcription from yeast promoters linked to consensus estrogen-responsive elements (ERE) in response to estradiol [22–27].

2. Materials and methods

2.1. Chemicals

The steroid compounds used in this study (E2, DES, 4-OHT, RAL and ICI) were purchased from Sigma (Paris, France) and solubilized in ethanol except for RAL and ICI which were solubilized in DMSO. The steroid stock solution concentration was 10^{-2} M.

2.2. Plasmids

The construction of expression plasmids of hERα and rtERα$_S$ has been previously described [24]. To construct expression plasmids of recombinant chimeric GFP–hERα and GFP–rtERα$_S$ proteins, the hERα and rtERα$_S$ cDNA were PCR-amplified from YEPE15 [28] and pCMV5/rterα$_S$ [29] respectively, and subcloned into a modified pBluescript SK phagemid (Stratagene). The subclonings of herα and rterα$_S$ were realized between EcoRI and SacI restriction sites. Resulting plasmids were designated as pBKherα and pBKrterα$_S$. Then GFP cDNA was amplified by PCR from pEGFP-N1 (Clontech, Palo Alto, CA) and subcloned into pBKherα and pBKrterα$_S$ between EcoRI and BamHI, upstream of the ER sequences. The unique BamHI site is present into the sense primers used for herα and rterα$_S$ amplification. The reverse primer used for GFP amplification has been designed so that the stop codon has been mutated from TAA to GGA in order to allow translation of the complete recombinant fusion proteins. Finally resulting plasmids pBKgfpherα and pBKgfprterα$_S$ were fused to pYeDP60-AQPcic [30] with SacI. Correct fusion plasmids were digested by EcoRI and the products were ligated in order to obtain the pY60gfpherα and pY60gfprterα$_S$ vectors.

Thus, all constructed plasmids contain the yeast ura and the bacterial amp^R selection markers. Additionally, in each case, expression of hERα or rtERα$_S$, fused or not with GFP, is under the control of a GAL10-cyc promoter and can be induced by addition of galactose (to a final concentration of 2% (w/v)) in the yeast culture medium.

β-galactosidase reporter vector, YRPE2, contains 2 consensus ERE sequences (ERE$_{cs}$) located upstream of a minimal cyc promoter controlling the lacZ gene expression [24]. This plasmid was kindly provided by Pr. O'Malley (Baylor College of Medicine, Houston, USA). The plasmid structures are available as Supplementary material.

2.3. Cell culture and transient expression assays

S. cerevisiae yeast cells, which lack endogenous estrogen receptors, were transformed with appropriate expression and reporter plasmids using the lithium acetate chemical method (Yeast protocols handbook, Clontech). The yeast *S. cerevisiae* strain W303.1B (α, leu2, his3, trp1, ura3, ade2-1, can^R, cyr+) was grown in rich medium YPRE (2% tryptone, 1% yeast extract, 0.5% raffinose, pH 7.0 and 3% ethanol) or selective medium (0.67% nitrogen base without amino acids, 2% glucose, pH 5.8, plus drop-out