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Regulation of transcriptional activation function of rat estrogen receptor α (ER α) by novel C-terminal splice inserts*

Pallob Kundu^{a,b,*}, Min Li^a, Rong Lu^a, Enrico Stefani^{a,c,d,e}, and Ligia Toro^{a,d,e,f}

^a Department of Anesthesiology, University of California, Los Angeles, CA 90095, USA

^b Division of Plant Biology, Bose Institute, Kolkata 700054, India

^c Department of Physiology, University of California, Los Angeles, CA 90095, USA

^d Department of Brain Research Institute, University of California, Los Angeles, CA 90095, USA

^e Department of Cardiovascular Research Laboratory, University of California, Los Angeles, CA 90095, USA

^f Department of Molecular and Medical Pharmacology, University of California, Los Angeles, CA 90095, USA

Abstract

Estrogen receptor α (ER α) mediates estrogen diverse actions on tissues. ER α gene has eight constitutively expressing exons and is known to have multiple isoforms generated by alternative initiation of transcription and splicing events including exon skipping. We have discovered two novel exons inserted between exon 5 and 6 of rat ER α that can add independently or in tandem 18 and 14 amino acids to the estrogen binding/activator function 2 domain of the receptor. Their transcript expression is three-to sixfold higher in heart compared to brain, aorta, liver, ovary and uterus. In heart, the new variants increased ~2 fold with animal growth from prenatal to adulthood, and had a minor increment in aged animals (28 months). Inclusion of these exons yields a receptor with practically no binding capacity for estrogen and reduced dimerization. The new variants show nuclear localization but are less efficient in binding to estrogen responsive elements (EREs) and failed to transcriptionally activate promoters containing EREs (mSlo, KCNE2). Thus, the new variants can regulate the wild-type receptor function and may contribute to the regulatory action of estrogen, especially in the maturing heart where they are more abundant.

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* Corresponding author. Division of Plant Biology, Bose Institute, P 1/12 CIT Scheme, VII M, Kolkata, West Bengal 700054, India. Tel.: +(91) (33) 2569 3298. pkundu00@gmail.com (P. Kundu)..

Appendix: Supplementary Material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2014.11.001.

Uncited reference

Dignam, J.D., Lebovitz, R.M., Roeder, R.G., 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11 (5), 1475–1489.

Keywords

Estrogen receptor; Alternative splicing; Steroid receptor coactivator; Transcription regulation; Heart

1. Introduction

Estrogen receptor alpha (ER α) is one of the most studied transcription factors. ER α and its counterpart ER β mediate a variety of actions that estrogen has on tissues in physiological as well as pathological conditions (Burns and Korach, 2012; Cui et al., 2013). Estrogen is critical for the development of female reproductive organs. In addition, it has significant effects on the central nervous system, bone development and the cardiovascular system. In the cardiovascular system, estrogen triggers protective mechanisms encompassing signaling cascades and expression of genes including ion channels or channel subunits (Kundu et al., 2007, 2008; Murphy, 2011). Various studies have depicted the complexity of ER α function and regulation of protein expression (Arnal et al., 2012).

The expression of ER α is regulated at transcriptional, posttranscriptional and posttranslational levels. At the transcriptional level, one inhibitory mechanism is promoter methylation. Vitamin D and progesterone are also inhibitory whereas estrogen has a stimulatory effect on its transcription. Alternative promoter choice, 5'-UTR selection also plays a significant role in the regulation of ER α gene expression (Ishii et al., 2010; Kobayashi et al., 2011). At the post transcriptional level, several micro RNAs (miRNAs) target ER α and coregulators (Klinge, 2012). miR-22, miR-221, miR-222, miR-18a,b, miR-193, miR-302c, miR-206 downregulate the ER α gene. Human ER α has two miR-206 target sites in its 3'-UTR. This miRNA represses hER α expression in breast cancer cell lines (Adams et al., 2007). Expression of some of these miRNAs is regulated by estrogen (Klinge, 2012), hinting at another level of regulation of ER α gene expression. At the posttranslational level, estrogen binding to ER α leads to its degradation via the ubiquitin-proteasome pathway (Lonard et al., 2000).

The function of the receptor can be regulated by posttranslational modifications including acetylation, S-nitrosylation and phosphorylation. Acetylation of the protein increases ER α transcriptional activity; S-nitrosylation inhibits ER α 's ability to bind to estrogen receptor elements (EREs); and MAP kinase dependent phosphorylation is important for ligand independent activation (Cui et al., 2013; Murphy et al., 2011). On top of these secondary modifications, several splice variants exert dominant negative effects on the functionality of the constitutive (labeled here wild-type) receptor. For example, naturally occurring splice variants including exon-skipping ER E3, ER E5, ER E7 and truncated ER product-1 (TERP-1, a unique 5'-untranslated region fused to exons 5–8 of ER α) have dominant negative effects on ERE containing promoters (Bollig and Miksicek, 2000; García Pedrero et al., 2003; Schreihöfer et al., 1999). Certain variants also showed a positive effect on WT ER α function. At low concentration, TERP-1 stimulated ER α -mediated activation of a model promoter (Schreihöfer et al., 1999). ER E3 variant activated a fragment of

ovalbumin promoter having only ERE half site and AP-1 sites. These stimulatory effects of variants on ER α activity were attributed to their interaction with coregulators.

Although the ER α gene expresses hundreds of different variants, their role in mediation of estrogen action is relatively unexplored. In the present study, we have found novel splice variants of rat ER α that have alternative exons inserted in the ligand binding/AF-2 domain of the receptor. These variants are mostly expressed in heart tissue, increase with development, and showed a dominant negative effect on transcriptional activation function of the wild-type receptor. The predominant expression in heart implies a potential role in fine tuning ER α function in this organ, in particular during development.

2. Materials and methods

2.1. Animals

Young adult (~3 months) Sprague–Dawley male or female rats, and young (3–4 months) and old (~24 months) F344 male rats were used. Prenatal hearts were collected from late pregnant Sprague–Dawley rats. Animal protocols were approved by UCLA, IRB.

2.2. Tissue collection

Tissues were collected immediately after sacrificing the animal and cleaned in cold phosphate buffered saline (10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4) prior processing.

2.3. Reagents

Trizol reagent (Invitrogen) was used for total RNA preparation. AMV RT was from Promega. Polymerases, restriction and modifying enzymes were from New England Biolabs, Qiagen and Invitrogen. Plasmids were prepared using Qiagen kits, and clones were sequenced with BigDye Terminator v3.1 (Applied Biosystems). 17- β -estradiol was from Sigma. Estradiol [2,4,6,7,16,17- $^3\text{H}(\text{N})$] (152 Q3 Ci/mmol), γ - ^{32}P labeled ATP and poly dI-dC or poly dI-dC were purchased from GE Healthcare.

2.4. Cell culture

HeLa (human epithelial) cells were purchased from American Type Culture Collection (ATCC). Cells were maintained in cell culture media consisting of Dulbecco's modified Eagle's medium (DMEM, high glucose, with L-glutamine) (Invitrogen) supplemented with 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal bovine serum under 5% CO_2 air mixture at 37°C. Cells were subcultured as recommended by ATCC.

2.5. Isolation of splice variants

Total RNA was prepared from whole hearts using Trizol reagent and purified using Qiagen RNeasy kit. The quality of the RNA preparation was checked by agarose gel electrophoresis. About 5 μg of total RNA was converted to cDNA using either OligodT $_{18}$ or a gene specific primer targeting ER α 's stop codon (ER-GSP-R) and Superscript III Reverse transcriptase. PCR amplification with gene specific primers (Table 1) was done using 10% of the cDNA product. Primers ER-ex1-F and ER-ex5-R were designed to detect splice variants derived

from the region in between exons 1 and 5. Similarly, primer pair ER-ex4-F and ER-ex8-R was used to detect the variants generated from genomic regions in between exons 4 and 8. Variants obtained in addition to the constitutive bands were gel purified and re-amplified with internal primer pair ER-ex4int-F/ER-ex8int-R. The nested PCR products were gel-purified and cloned in TA-vector for sequencing (GenBank KJ746832 and KJ746833).

2.6. Plasmid constructs

All constructs were made using standard recombinant DNA techniques. Promoter-reporter constructs were generated by subcloning promoter regions of mSlo (−4910/+7) and KCNE2 (−951/+ UTR) genes (Kundu et al., 2007, 2008) in promoterless pGL3-Basic and pGL4.1 vectors (Promega), respectively. Full length ER α was amplified from cDNA prepared from SD rat uterus total RNA and cloned in HindIII and ApaI sites of pcDNA3.1 vector (Invitrogen). The cloned sequence was similar to the previously reported mRNA (GenBank X61098). Splice variant sequences were inserted to this sequence in between exons 5 and 6.

To make C-terminal GFP-tagged rat(r) ER α s both WT and rER α C-terminus splice variants (rERCsvs) were PCR amplified using primers
GCGCAAGCTTGCCACCATGACCATGACCC.

TTCACACC and TTTTGGTACCGCGAT GGTGTTGGGGAAGCCCTC. PCR products were cloned into HindIII and KpnI sites of pEYFP-N1 and pECFP-N1 vectors. Clones were checked by sequencing. Protein expression efficiency was analyzed by Western blotting of lysates from transfected HeLa cells.

2.7. Reverse Transcription (RT) and real-time PCR

About 5 μ g of total RNA isolated from various tissues was converted to cDNA using either OligodT₂₀ or random hexamer primers and Superscript III Reverse Transcriptase (Invitrogen). Real-time PCR reactions (50 μ l) were done with 5% of the RT reaction product using iQ SYBR green mix and the iCycler iQ (BioRad). Gene-specific primers were used to detect various forms of ER α . Forward primers ER-ex5_6F, ERCsv1-F, ERCsv2-F and ERCsv1-2-F in combination with the reverse primer ER-ex6-R were used to detect insertless, rERCsv1 + rERCsv1-2, rERCsv2 + rERCsv1-2 and rERCsv1-2, respectively. Expression of the constitutive form of rER α was detected with ER-ex7_8-F and ER-ex8int-R primer pairs that amplified a region extending from the junction of exons 7 and 8 to an internal region of exon 8. Relative expression levels were determined using the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak, 2008). Controls included the mock cDNA (reverse transcriptase-independent), no-template control (H₂O control) and transcript levels of the housekeeping gene, rat GAPDH. Specific products were detected as clear single peaks at their melting temperature in the first derivative of fluorescence (dF/dT) versus temperature plot (melting curve) and by agarose gel electrophoresis of PCR end-products. All experiments were done in triplicate using three independent RNA preparations.

2.8. Estrogen binding assay

Wild type and mutant ER α proteins were synthesized *in vitro* using the rabbit reticulocyte lysate translation mix (TnT mix, Promega). For each protein, the synthesis reaction (100 μ l)

contained 80 μ l TnT quick master mix, $\sim$ 2 μ g of rER α or rERCsvs DNA (in pcDNA3.1) and 0.02 mM Methionine. The control reaction contained pcDNA3.1 plasmid instead of rER α clones. Expression of rER α proteins was confirmed in parallel by metabolic labeling using 10 μ l of the synthesis reaction and 5 μ Ci of [35 S]-L-methionine ([35 S]-Met) (>1000 Ci/mmol at 10 mCi/ml, MP Biomedicals or Perkin Elmer) instead of unlabeled methionine. Reactions were incubated at 30 $^{\circ}$ C for 1.5 h and transferred to -20 $^{\circ}$ C if not immediately used. Only 2 μ l of this reaction was separated in 10% SDS-PAGE and radiolabeled products were detected using autoradiography.

A typical binding reaction had 10 μ l of the synthesis product, 2 μ Ci [2,4,6,7,16,17- 3 H]Estradiol (GE Health Care, 152 Ci/mmol, 1 mCi/ml) and 88 μ l of Buffer D (20 mM HEPES, pH 7.4, 10% Glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 1 mM DTT). Binding reactions were carried out at room temperature for 3 h. Estradiol-bound proteins were then separated from free radiolabeled estradiol by purification through Sephadex G-25 spin columns (Roche or GE Health Care) equilibrated with Buffer D. Eluates containing radiolabeled estradiol-bound proteins were mixed with 5 ml of scintillation cocktail and counted using a liquid scintillation counter (Beckman). The amount of bound estradiol (in fmol) was calculated based on the specific activity of the added [3 H]-estradiol.

2.9. Electrophoretic mobility shift assay (EMSA) and reverse EMSA

ER α proteins were synthesized by *in vitro* transcription/translation in rabbit reticulocyte lysate as described above. This reaction was carried out in the presence of 1 nM estrogen to facilitate dimerization of the protein. ERE consensus and Sp1 duplexes (containing binding site for Sp1 transcription factor) were from Santa Cruz Biotechnology and Promega Inc., respectively. Sense and antisense oriented oligonucleotides spanning KCNE2 mutated ERE (Kundu et al., 2008) were synthesized and annealed to make a duplex probe. ERE duplexes ($\sim$ 3 pmol) were end-labeled with polynucleotide kinase and γ - 32 P[ATP]. The labeled probe was purified using Quick spin oligo columns (Roche) and $\sim$ 50,000 cpm ($\sim$ 3 fmol) of the probe was used in a 15 μ l binding reaction. The binding reaction was performed in a binding buffer containing 5% glycerol, 20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 2 mM MgCl $_2$, 0.5 mM PMSF, 1 mM DTT and 1.5 μ g poly (dI-dC)Opoly(dI-dC). Only 1 μ l of the *in vitro* protein synthesis reaction mix containing ER protein was used in this reaction. The protein was preincubated in binding buffer for 5 min prior to the addition of probe, and incubated for another 20 min at room temperature. DNA-protein complexes were resolved in a 0.5 X TBE-4% PAGE (10 $^{\circ}$ C, 220V) for about 2 h and processed for autoradiography.

To perform reverse EMSA, 35 S-Met-labelled ER (wild-type and variants), and control proteins (5-HT $_2$ A R) were synthesized *in vitro* as described for the estrogen binding assay. During synthesis of the protein, 10 nM estrogen was added to the reaction to promote dimerization of the protein. EMSA reaction was carried out in binding buffer containing 1.5 pmol of unlabelled ERE duplex and 1 μ l of the *in vitro* protein synthesis mix. Reactions were carried out at RT for 20 min and immediately loaded in a 5% Polyacrylamide-0.5X TBE gel without adding any other buffer or dye. Gel was run and processed as described in the previous paragraph.

2.10. Fluorescence microscopy

HeLa cells were seeded on coverslips previously coated with 0.1 mg/ml Poly-D-Lysine + 0.1 mg/ml Collagen for 3 h and washed two times. Cells were cultured in DMEM without phenol red for about 18 h to achieve 50% confluence prior to transfection with ER α constructs using Lipofectamine 2000 reagent. Transfection was carried out in presence of 10% serum and 1 nM estrogen. About 24 h post-transfection, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA, in phosphate buffer) for 20 min at room temperature, and washed three times with PBS for 5 min each. Blocking was done with 10% Normal Goat Serum in PBS + 0.2% Triton X100 for 30 min. Immunostaining was done with anti-rat ER α antibody MC-20 (0.6 μ g/ml) and Alexa Fluor 488 Goat anti-rabbit IgG (2 μ g/ml) secondary antibody following standard protocols (Roper et al., 2006). IgG isolated from normal animals was used instead of primary antibody (negative control). Finally cells were mounted with Prolong anti-fade kit (Molecular Probes). Cells transfected with fluorescence protein tagged ER α s were mounted after fixing. In all experiments cells were visualized with an Olympus Fluoview confocal microscope.

2.11. Cell transfection and Luciferase assay

About 50–70% confluent cells grown in 24-well plates were transfected with promoter-luciferase (mSlo or KCNE2 or 6XERE-reporter; SuperArray Bioscience) constructs or empty vector as control using Lipofectamine 2000 reagent following manufacturer's instructions (Invitrogen). pRL-TK vector, which directs Renilla Luciferase (RLuc) constitutive expression, was used in all experiments as the transfection control to normalize Firefly Luciferase (Luc) activity. Transfection was carried out for 16 h in serum-free media, followed by regular culture for 30 h prior cell lysis.

For experiments with ER α s, cells were cotransfected with rat estrogen receptor α (rER α) or rERSV expressing vectors. Unless otherwise stated, rER α DNA was 0.125 times the concentration of the promoter constructs. Phenol red free media was always used and transfection was carried out overnight (16 h) in serum-free media. Next day, cells were induced with estradiol (1 nM to 10 nM) or carrier (ethanol < 0.01%) in cell culture media but with charcoal-dextran stripped serum (HyClone). Luciferase assays were performed 24 h after induction. Results are presented as average of at least three transfections.

Cells were lysed with passive lysis buffer and assayed using Promega dual luciferase assay kit and the Veritas luminometer (Turner Biosystems). Results are expressed as Luc/RLuc ratio. In experiments with constructs of different sizes equal molar amount of DNA was transfected and the total amount of DNA transfected was kept constant using the pGem3Zf-vector.

2.12. Fluorescence Resonance Energy Transfer (FRET) analysis

HEK293 cells were grown in clear bottom 96-well plates to about 60% confluence. Cells were transfected with ER α isoforms fused to CFP or YFP in different combinations. As positive control, cells were transfected with CFP-linker-YFP construct (linker consisting of 6 aa). For negative control, cells were co-transfected with pEYFP-N1 and pECFP-N1 vectors to individually express EYFP and ECFP. In all experiments, an equal molar amount

of each construct was used and the total amount of DNA was kept constant by adding pcDNA3.1. Transfection was carried out with Lipofectamine 2000 in 100 μ l DMEM (without phenol red) supplemented with 10% charcoal-dextran stripped serum, nonessential amino acids (NEAA) and 10 nM estrogen. After 36 h, media was replaced with PBS. FRET analysis was done using the Synergy 2 Multi-Mode Microplate Reader (Biotek) using the bottom reading mode. Filter sets used for measuring CFP, YFP and FRET fluorescence values were 400 $\pm$ 10 nm, 460 $\pm$ 40 nm; 485 $\pm$ 20 nm, 540 $\pm$ 25 nm, and 400 $\pm$ 10 nm, 540 $\pm$ 25 nm for excitation and emission wavelengths, respectively. Net FRET values were determined after subtracting the bleed through values of CFP and YFP from the reading at the FRET channel.

2.13. Statistics

All experiments were performed with three different RNA preparations or three different batches of transfected cells. For real-time PCR analysis, reactions were also done in triplicates. Results are expressed as means $\pm$ standard error (SE). Two-tail Student's *t*-test was used and $P < 0.05$ was considered significant.

2.14. Software

Homology search and alignments were done using NCBI BLAST, NCBI ALIGN and CLUSTALW2 tools. Protein domains were predicted using EXPASY tools.

3. Results

3.1. Detection and cloning of ER α splice variants from rat heart

Figure 1A shows the exonic composition of constitutive (wild-type) ER α highlighting the position of functional domains. To search for splice variants, we used an exon scanning PCR strategy (primers are given in Table 1 and Fig. S1A). To this end, total RNA of 3-month-old male SD rat heart was converted to cDNA using either oligodT or a gene specific primer (ER-GSP-R) targeting sequences near ER α 's stop codon. ER α exons were PCR amplified in two sections covering the entire ER α coding sequence; exons 1 to 5 (primer pair ER-ex1-F/ER-ex5-R) and exons 4 to 8 (primers ER-ex4-F/ER-ex8-R). Products without splicing inserts were expected to have 858 and 826 nt, respectively. Analysis of amplified products using agarose gel electrophoresis yielded in addition to the expected products, two bands of higher molecular size migrating just above the 826 nt band that could be amplified from cDNAs prepared either with OligodT or ER-GSP-R primers (Fig. S1B). The extra bands were purified and subjected to nested PCR with internal primers also targeting exons 4 to 8 (ER-ex4int-F/ER-ex8int-R). Sequencing of the nested PCR products revealed two novel splice inserts in between exons 5 and 6 (Fig. 1B), named here, rERCsv1 and rERCsv2 (rat ER α C-terminus splice variant 1 and 2). rERCsv1 insert could be detected independently or in tandem with rERCsv2 (named as rERCsv1-2) (Fig. S2). To confirm that these splice inserts were part of the full length CDS of rER α , a region spanning from the start codon to almost the end of the gene was PCR-amplified with primer pairs ER-ATG-F and ER-ex8-R using cDNA prepared with OligodT; the PCR product was then subjected to internal PCR using ER-ex4int-F/ER-ex8int-R primers. Using this strategy, we were able to reproduce amplification of the two extra bands, confirming the inclusion of these splice inserts as part

of the full length rER α . Analysis of available rat genomic data also confirmed their existence as exons in the intronic region between exons 5 and 6 as the inserts are flanked by sequences that are hallmark of intron boundaries (Supplementary document 1).

Splice inserts rERCsv1 and rERCsv2 add in frame 18 aa (54 nt) and 14 aa (42 nt) respectively, to ER α within its ligand binding/ AF2/SRC interacting domain.

3.2. Spatio-temporal expression pattern of novel variants

We first examined whether the new splice variant transcripts detected in heart were present in other tissues as well. To this end, real-time PCR analysis was performed in aorta, brain, liver and uterus along with heart tissues. Primers used in the detection of rERCsv1 and/or rERCsv1-2 are indicated in Fig. 2A and B (inset) and Table 1. The primer pair F1 (ERCsv1-F) and R1 was designed to detect both rERCsv1 and rERCsv1-2; the pair F2 (ERCsv1-2-F) and R1 was designed to specifically detect rERCsv1-2; while the pair F3 (ERCsv2-F) and R1 would detect insertion of rERCsv2 and rERCsv1-2. To detect expression of total ER α , F4 (ER-ex7_8-F) forward primer was designed at the junction of constitutive exons 7 and 8 and used with reverse primer R2 (ER-ex8-R). A typical agarose gel electrophoresis of PCR end-products are shown in the inset to Fig. 2B. Expression levels of splice variants were calculated as the percentage of splice variants present with respect to total rER α for each tissue examined. Figure 2B shows that, in the rat, splice variants are mostly expressed in heart where expression levels of rERCsv1 and rERCsv1-2 variants could be as much as 6% of total ER α . In contrast in the brain, expression levels were up to 2% of total ER α . Expression levels of rERCsv2 and rERCsv1-2 variant or rERCsv1-2 alone was less than 2% in all tissues.

Next, we determined the effect of age on variant expression levels in the heart. For this purpose, we collected total RNA from prenatal, young (3–4 months old) and old (~24 months old) hearts and followed a similar protocol to the one described above but normalizing to insertless product. The insertless fraction was determined by using F5 (ER-ex5_6-F (F5) forward primer spanning the junction between exon 5 and 6 and reverse primer R1. Figure 2C shows that splice insertion of rERCsv1 and rERCsv1-2 increases with age reaching as high as 13.3% of the insertless ER α in old rat heart. The differences between young adult and old rat heart with respect to prenatal samples were significantly different for all forms of variants. From adult to old; however, there was a tendency to increase that was not significant. Thus, splice insertion of the new variants is regulated as an age-dependent process that stabilizes during adulthood.

3.3. Biochemical and functional characterization of splice variants

Because the novel exons were spliced in the ligand binding domain of ER α (Fig. 1A), which is also important for its interaction with steroid receptor coactivators and contains the activation function 2 (AF2) domain, we decided to determine whether splice variants modified the properties of the receptor. Properties examined were: (i) ligand binding, (ii) homo and hetero dimer formation, (iii) ERE binding, (iv) nuclear localization, and (v) transcriptional activation of known estrogen responsive promoters.

- (i) Ligand binding assay. The assay was carried out with [2,4,6,7,16,17-³H] Estradiol and *in vitro* synthesized ERα proteins. Figure 3A shows that wild-type (WT) ERα was able to efficiently bind estradiol in a dose-dependent manner. In contrast, the binding efficiency of splice variants, rERCsv1 and rERCsv1-2, was greatly reduced irrespective of the amount of the hormone. At 10 nM estrogen, rERCsv1 and rERCsv1-2 showed only 11% and 5.5% efficiency in estrogen binding compared to the WT receptor (n = 2). The inset shows [³⁵S]-Methionine labeled WT and splice variant ERα proteins labeled in parallel demonstrating that equivalent amount of proteins were used for the binding assay.
- (ii) Homo- and hetero-dimer formation efficiency. To determine the dimerization efficiency of splice variants with respect to WT ERα, we utilized a FRET assay. C-terminus CFP and YFP fused constructs for WT and both splice variants, rERCsv1 and rERCsv1-2 (Fig. 3B), were generated and checked by sequencing. The constructs were first tested for their activeness by analyzing their promoter activation function utilizing a luciferase reporter containing six EREs and stimulating activity with 1 nM estrogen. WT-fluorescent protein fusion constructs displayed fairly equivalent promoter activation to non-tagged WT constructs (Fig 3B); while tagged splice variants were almost unable to activate promoters irrespective of the type of fused fluorescent protein (not shown). This property was not due to the fusion of CFP or YFP as untagged splice variants were also unable to drive promoter activation (see Fig. 6). Therefore, we proceeded to measure their ability to dimerize. WT-CFP and WT-YFP FRET value was considered as 100% homo-dimerization. The results showed that splice variants were less efficient in forming homodimers. Interestingly, both variants were able to hetero-dimerize with the WT ERα to different degrees. While rERCsv1-YFP was able to almost fully heterodimerize with WT ERα, the heterodimerization efficiency of rERCsv1-2-YFP was lower but significant and was ~66% (Fig. 3C; n = 4 for each pair).
- (iii) ERE binding. Wild type ERα protein is known to bind to ERE sequences via its DNA binding domain. To test if the new splice variants share this ability, gel mobility shift assays (EMSA) were performed using 5'-labelled DNA duplexes containing a perfect ERE, a mutated ERE (derived from KCNE2 gene) and an SP1 binding sequence (Fig. 4A) along with non-radiolabeled WT ERα and rERCsv proteins expressed *in vitro*. The EMSA assay revealed that WT and rERCsv1 isoforms were able to interact with ERE consensus DNA duplex; however, ERE interaction with rERCsv1-2, if any, was not detectable (Fig. 4B). The assay also showed that rERCsv1 binds to the ERE less efficiently compared to the WT ERα. As negative control, all ERα isoforms tested were unable to interact with the mutated ERE duplex (gel at right), further confirming the specificity of the interaction between rERCsv1 and ERE. To validate the above finding, reverse EMSA assays with ³⁵[S]-labeled proteins and non-labeled DNA duplexes were performed (Fig. 4C). Here, we also found that WT ERα and to a lesser extent rERCsv1 were able to interact with ERE DNA duplex. Consistent with the EMSA result, we could not detect DNA-protein complex of rERCsv1-2

and ERE duplex. Absence of shifted bands using Sp1 DNA duplex (left gel) or with no-DNA (negative control) confirmed that the detected interaction was specific.

- (iv) **Nuclear localization.** To determine whether the new splice variants affect ER α 's subcellular distribution, two complementary studies were carried out using transfected HeLa cells: a) Immunofluorescence using ER α antibody; b) imaging of YFP-tagged ER α isoforms. In the immunofluorescence study, we used as negative controls, WT ER α transfected cells analyzed with IgG or nontransfected cells treated with anti-ER α antibody. In both cases, we did not observe signals above background (Fig. 5A, first two panels), supporting the specificity of the antibody used. In contrast, examination of transfected cells labeled with anti-ER α clearly showed that both WT and splice variants were primarily localized in the nucleus (number of cells examined = 15 cells minimum for each condition). This result was directly confirmed by visualizing HeLa cells transfected with YFP tagged proteins. The fluorescent images also showed that WT-ER α and splice variants mostly localized in the nucleus (Fig. 5B). However in this case, the rERCsv1-2 protein was also evident in the cytoplasm.
- (v) **Transcriptional activation of known estrogen responsive promoters.** To test whether splice variants have normal transcriptional activity, we used two ER α responsive promoters belonging to mSlo and KCNE2 genes, and luciferase as a reporter (Kundu et al., 2007, 2008). HeLa cells were transfected with each of the promoter-luciferase constructs along with rat ER α bearing plasmids. Luciferase activity was monitored after treatment of transfected cells with different doses of estrogen. In the presence of ER α , mSlo promoter activity was significantly increased by estrogen reaching a maximum at 1 nM. Similarly, KCNE2 promoter was also induced by 1 nM estrogen when ER α was coexpressed. However, both splice variants were unable to induce any mSlo or KCNE2 promoter activity at all estrogen concentrations tested (for mSlo promoter, up to 100 nM) (Fig. 6A, B). Next, we tested if this 'inactivity' of splice variants could be compensated by increasing the amount of receptor isoform being cotransfected. Figure 6 shows that splice variants remained without activity regardless of the amount of transfected DNA. This was in contrast to wild-type ER α , which showed increasing mSlo promoter activation with increasing DNA concentration. Taken together, the results indicate that splice variants lack transcriptional activation function that can be explained by their limited (or lack thereof) estrogen binding, dimerization and ERE binding abilities.

3.4. Effect of splice variants on activation function of wild-type ER α receptor

As splice variants are likely co-expressed in a native cell along with WT receptors, we next investigated whether the expression of rERCsv1-2 variant has an effect on the transcriptional activation function of WT ER α . We chose this variant because it displayed reduced heterodimerization with WT receptor. To this end, we examined mSlo (Fig. 7A) and KCNE2 (Fig. 7B) promoter activity in cells transfected with varying ratios of WT

ER α :rERCsv1-2 DNAs and stimulated with 1 nM estrogen (n = 4). As control, we used WT:empty vector DNA; and the total amount of receptor mix was kept comparable between the control and the experimental set. The results revealed that as little as 10% of rERCsv1-2 showed a tendency to negatively affect the function of WT ER α resulting in decreased mSlo and KCNE2 promoter activity. This downregulatory effect was statistically significant when 50% (panel A)/80% (panel B) of the Q5 transfected DNA was from rERCsv1-2.

4. Discussion

In the rat genome, the ER α gene (*Esr1*) is found in chromosome 1 (NCBI Gene ID 24890, NC_005100.3, 42667185 to 42931332; Rat genome database (RGD) ID 2581). The new splice variants reported here -rERCsv1 and rERCsv2- are located within the intronic region of exons 5 and 6; their gene location coordinates are 42894930 to 42894983 and 42895975 to 42896016, respectively. Both alternative exons are flanked by sequences characteristic of splice acceptor and donor sites (Supplementary document 1). Accordingly Internet based softwares, Berkeley Drosophila Genome Project (BDGP) and SplicePort, accurately predicted boundaries of these two exons. Other than these two splice inserts, we could not detect any additional insertions in rER α at this site in cardiac mRNAs. Our exon-scanning screening resulted in the exclusive detection of insertions of rERCsv1 and rERCsv1-2; while rERCsv2 insertion alone was not detected. However, we cannot rule out insertion of this variant alone because of the low number of cloned colonies that were screened (about 15). Our quantitative RT-PCR analysis indicated that transcript expression of this form of the variant, which would generate an in-frame protein, is minimal (compare rERCsv2 and 1-2 vs. rERCsv1-2 expression levels; Fig. 2B, C). In addition to these splicing inserts between exons 5 and 6, other previously detected deletion variants missing exons 4 or 5 or 6 or 7 (Friend et al., 1997; Horvath et al., 2002; Marshburn et al., 2004) were also found in our analysis. Two new deletion variants, one omitting three exons (exons 5, 6 and 7) and another with partial deletion of exon 8 were also noticed. Although the functional significance of the splice variants identified here remains to be elucidated, it is interesting to note that insertion of rERCsv1-2 introduces two additional PKC phosphorylation sites (SER and SLR) to the receptor. This type of phosphorylation has been linked with ER α degradation via ubiquitinproteasome pathway (Johnson and O'Malley, 2011; Marsaud et al., 2003) and ER α phosphorylation signature is altered during breast cancer development (Murphy et al., 2011).

The new ER α variants were detected in total RNA isolated from young-adult rat heart tissue. Analysis of their expression pattern in other tissues indicated that the expression of these splice variants is maximum in rat heart. This expression pattern was independent of the expression level of the constitutive form of ER α . The results also indicated that the expression of these variants in the heart is age-dependent and that their expression level was highest in the old hearts. Consistent with our results, in the brain, expression of other variants namely ER α 7 and 2 variant is age dependent; in this case, expression was also region-dependent (Ishunina and Swaab, 2008). A recent report described differential expression of splice variants in zebrafish heart, although their expression was not age dependent (Cotter et al., 2013). Therefore, our work further supports the view that the spatio-temporal expression of ER α splice variants may contribute to the regulatory role of the receptor.

The splicing position of the new exons predicted to disorder the receptor's ligand binding domain and one part of its dimerization domain (Fig. 1). Accordingly, it was found that the new variants were almost incapable of binding estrogen, and consequently, estrogen dependent dimerization was negatively affected in such a way that homodimerization of variants was less efficient than when variants were allowed to heterodimerize with WT ER α (Fig. 3).

Although the nuclear localization signal or DNA binding domain were away from the insertion site, splice inserts seemed to exert long-range effects on these receptor characteristics. Specifically, rERCsv1-2 besides being expressed in the nucleus could also be located in the cytoplasm (Fig. 5B) and did not bind to ERE (Fig. 4). Moreover, ERE recognition efficiency was inversely related to the length of the insertion following the sequence WT ER α > rERCsv1 > >>rERCsv1-2 (Fig. 4). Therefore, it can be safely assumed that these new splice insertions can create structural constraints affecting the DNA binding domain of the receptor.

The variants were found to be transcriptionally inactive (Fig. 6); in addition, rERCsv1-2 had a dominant negative effect on the activity of the WT receptor (Fig. 7). This effect achieved significance when the amount of expressed splice variant (50% of the total assuming equal efficiencies of transfection/transcription) was much more than what is predicted from its relative mRNA abundance (~2%) (Fig. 2). Thus, it is tempting to suggest that within 'normal' conditions rERCsv1-2 may fine tune the activity of ER α and that an increased expression of the variant would lead to decreased ER α activity.

Inhibition of ER α activity is an important modulatory process by which the function of this receptor is regulated in a spatio-temporal manner. This is achieved naturally by either expression of dominant negative isoforms of interacting heterologous partners (Kumar et al., 2002 and Yamashita et al., 2003) or homologous partners (ER α variants). Several exon-skipping splice variants of ER α have been reported (Bollig and Miksicek, 2000; García Pedrero et al., 2003) that may have negative effects on ER α -mediated gene expression. Here, we have presented the first report showing a splice insertion in the ligand binding/activation domain generating a dominant negative isoform of the receptor, adding further complexity to ER α -mediated gene regulation.

A previously reported exon-skipping variant of ER α (ER E3) lacking only DNA-binding ability had distinct transcriptional effects depending on the promoter. When tested on a promoter containing a canonical ERE like motif, a negative effect on WT ER α transcription was observed; while when tested on a promoter with ERE half-site and an AP-1 motif, recruitment resulted in upregulation of promoter activity. Another exon-skipping variant, ER E5, had a dominant negative effect on WT ER α (Bollig and Miksicek, 2000). Our observations also fall within the same type of inhibition (Fig. 7); however, the cardiac variants are of a different kind, that is, they are products of spliced exons rather than of exon-skipping. In our work, we tested two promoters, the KCNE2 promoter that is active in heart (Jiang et al., 2004 and Abbott, 2012) and has only near canonical ERE sites, and the mSlo promoter that has EREs, 1/2EREs, AP-1 and Sp1 sites. In both cases, WT-ER α -mediated transcriptional activity was inhibited by rERCsv1-2 splice variant. These results

indicate that rERCsv1-2 can downregulate WT ER α transcriptional activation of promoters with different characteristics.

Analogous to our studies, ER β 2 sequence is known to carry a similar in-frame splice insertion at its ligand binding domain in rat and humans yielding inserts of 18 and 26 amino acids, respective-Q6 ly (XM_006240221, Fig. S3). These insertions also exhibit a dominant negative effect on the function of ER β (Chu and Fuller, 1997, Moore et al., 1998 and Hanstein et al., 1999). Interestingly, the rat variant has been linked with the effectiveness of estrogen replacement therapy (Wang et al., 2012).

Analysis of the human EST data set revealed that the human ER α gene also has a spliced exon in between exons 5 and 6 that yields an in-frame spliced insert of 23 aa (69 bp; EST clone S82164.1) (Murphy et al., 1996). The presence of this sequence was confirmed in the human genome; further, it is surrounded by quasi-perfect splice donor and acceptor sequences (Supplementary document 1). In the mouse, although rERCsv1 and 2 homologous sequences are found in the intronic sequence of exons 5 and 6, they are not flanked by donor/acceptor sequences and thus, they do not qualify to be exons (Supplementary document 1). The putative absence of a spliced exon(s) capable of disrupting the ER α ligand binding domain in the mouse in contrast to their presence in rat and humans supports the idea that having a splice insert in this position (Fig. S3) is a strategy acquired during evolution of the gene to create an extra level of regulation for the activity of the receptor.

Both dominant negative and positive isoforms are expressed from human ER α gene (Ishunina et al., 2013). In the human brain 62 different variants have been found, and among these a dominant negative deletion isoform, 7, was the major variant (Ishunina and Swaab, 2012). Transcriptional regulatory function of human splice variants differs based on the cell type and in cases of the variant TADDI (small deletion between exons 3 and 4 with an insert) even opposite effects have been observed in HeLa and M17 cell lines (Ishunina et al., 2013). hER α C-terminus splice variants lacking either exon 5 or exon 7 and having a dominant negative effect were found throughout the estrous cycle in the endometrium (Marshburn et al., 2004). hER α -46 and hER α -36 are also dominant negative isoforms with the potential of altering the non-genomic action of the receptor (Wang et al., 2005).

In microarray based genome wide exon junction analysis, it has been estimated that at least 74% of human multi exon genes are alternatively spliced (Johnson et al., 2003; Kornblihtt et al., 2013). These spliced isoforms play a significant role in the fine regulation of gene expression and an imbalance in their expression pattern might lead to disease progression (Tang et al., 2013). The human version of the splice insertion variant found in a similar location to rERCsv was from a breast cancer biopsy sample (Murphy et al., 1996). Several other hER α splice variants are differentially regulated in diseased tissues (Cui et al., 2013; Ishunina and Swaab, 2008, 2009, 2012; Miceli et al., 2011; Witek et al., 2007; Xing et al., 2013) and under different physiological conditions in a tissue-specific manner (Ishunina and Swaab, 2008). Moreover, in genetic association analysis hER dominant negative variants have been linked with schizophrenia (Weickert et al., 2008). Thus it has been postulated that altered expression of ER α splice variants might be linked with human patho-physiological changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ERα	estrogen receptor alpha
SRC	steroid receptor coactivator
WT	wild type
ERE	estrogen responsive element
rERCsv	rat estrogen receptor C-terminus splice variant

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Fig. 1.

Sequence of the new splice variants of rat estrogen receptor α (rER α). A, Diagram of rER α protein exonic composition and functional domains. Dashed lines mark the insertion site of the new splice variants in between exons 5 and 6. The two splice inserts of 18 aa (rERCsv1) and 14 aa (rERCsv2) long are depicted in different shades. rERCsv1-2, expression of both inserts in tandem; NLS, nuclear localization signal; DNA binding, ERE (estrogen responsive element) binding site; Ligand binding, estrogen binding site; AF2, activation domain for AF2; and SRC, steroid receptor coactivator interaction site. B, Nucleotide sequences of rERCsv1 and rERCsv2 (italics, underlined) along with partial sequences of flanking exons of rER α . C, rERCsv1 and rERCsv2 protein along with partial amino acid sequences of flanking exons.

Fig. 2.

Tissue specificity and age-dependent expression of rat ER α splice variants. A, Diagram showing exons 5 through 8 of rER α along with the new splice inserts. Arrows mark the position of primers (Table 1) used in the specific amplification of gene fragments. B, Relative expression levels of splice variants in different tissues of 3-month-old SD rats as determined by RT-dependent real-time PCR analysis. Aorta, brain, heart, and liver were from male rats. Splice variant levels were expressed as percentage of the expression of total ER α . Top inset, table of primer pairs and corresponding ER α products; bottom inset, agarose gel electrophoresis of PCR end-products shows bands of the expected size (235 bp, for total; 117 and 159 bp for rERCsv1 and 1–2; 96 bp for rERCsv2 and 1–2 and 129 bp for rERCsv1-2). C, Age-dependent expression levels of splice variants in rat heart. Relative amount of splice variants expressed in heart tissue of prenatal (SD rats), young (3–4 months) and old (~24 months) F344 male animals were analyzed using RT-dependent real-time PCR. Melting point curves and sizes of F344 PCR products were identical to the products amplified from SD rat sequences. Data are expressed as the percentage of expression relative to the insertless isoform. To measure the amount of insertless ER α , we used a forward primer overlapping the junction of exons 5 and 6 (panel A, F5) and a reverse primer within

exon 6 (panel A, R1). Sequences of all primers are given in Table 1. *, significantly different from prenatal samples.

Fig. 3.

Estrogen binding and dimerization efficiencies of splice variants. A, Estrogen binding efficiency of splice variants measured using [^3H]-estradiol. Inset, autoradio-gram showing equal expression of [^{35}S]-Methionine labelled WT ER α and variant proteins. B, Scheme of ER α -CFP and ER α -YFP C-terminal fusion proteins. CFP, Cyan fluorescence protein and YFP, yellow fluorescence protein. Bar plot shows the efficiency of WT-CFP and WT-YFP ER α in activating a 6X ERE promoter-luciferase reporter gene. Reporter plasmids were transfected in HeLa cells along with receptors at 0.1 $\mu\text{g}/\text{ml}$ transfection mix (reporter:receptor = 1:0.125). C, Comparison of dimerization efficiency of different ER α isoforms measured by FRET analysis. For each tagged receptor 3 μg plasmid DNA/ml transfection mix was used. Results are expressed as % of the dimerization efficiency of WT-ER α . *, significantly different from WT homodimerization efficiency.

Fig. 4. DNA-binding ability of ER α splice variants. A, Sequence of DNA duplexes used in EMSA assays. Dotted box, delineate the consensus ERE sequence in the duplex DNA; italicized and underlined letters, mutated bases to render the ERE unable to bind ER α . Lowercase letter indicates mismatch from consensus ERE. B, Autoradiogram of *in vitro* synthesized [35 S]-Methionine labeled ER α proteins resolved by SDS-PAGE. C and D, Autoradiograms of the electrophoretic mobility shift assay (EMSA) (C) and reverse EMSA (D) gels showing binding of WT and rERCsv1 ER α with the consensus ERE duplex DNA (arrow). rERCsv1-2 did not bind to the ERE. None of the proteins physically interacted with the mutated ERE of KCNE2 (C, right panel) or Sp1 binding site (D, left panel).

Fig. 5.

Subcellular localization of splice variants. A, Images of HeLa cells transfected or not with ER α constructs and immunolabeled with either a control immunoglobulin or with anti-ER α polyclonal antibody. Constructs (transfected at 2 μ g DNA/ml) and antibodies are indicated above the images. B, Nuclear localization of ER α s YFP-tagged expressed in HeLa cells (transfected with 4 μ g plasmid/ml). Each image is the sum of multiple confocal scans of different planes.

Fig. 6.

Transcriptional activation function of splice variants. A and B, Estrogen inducible promoter activity of known estrogen responsive promoters of mSlo and KCNE2 genes in the presence of WT ER α and splice variants. Cells were transfected with 0.8 μ g reporter plasmid DNA/ml and 0.1 μ g receptor plasmid DNA/ml. C, ER α -dose-dependent activation of mSlo promoter. Splice variants rERCsv1 and rERCsv1-2 failed to transcriptionally activate the promoter even in the presence of a relatively high amount of DNA used to transfect cells.

Fig. 7.

Splice variants modify the functional activity of WT-ER α . Estrogen (1 nM)-induced transcriptional activity of mSlo (A) and KCNE2 (B) promoters in the presence or not of WT ER α and rERCsv1-2 splice variant measured by the luciferase reporter assay. Cells were cotransfected with 0.8 μ g reporter plasmid DNA/ml and with varying ratios of WT:rERCsv1-2 DNAs as indicated in the figure. The total amount of plasmid DNA was kept constant using pGem3 vector.

Table 1

Primers used in this study. All primers were designed based on the sequence of rat ER α gene (X61098) and rat GAPDH gene (XM_573217).

Name	Primer sequence	Orientation	Detection
ER-GSP-R	TCTCAGATGGTGTGGGGAAG	Reverse	cDNA
ER-ATG-F	ATGACCATGACCCCTTCACACC	Forward	Full length
ER-ex1-F	CCAGTCCGCTGATGCTGCT	Forward	Splice variants
ER-ex5-R	GACCAGACCAATCATCAGGATCTC	Reverse	Splice variants
ER-ex4-F	GATGGTCAGTGCCTTATTGGATGCTG	Forward	Splice variants
ER-ex4int-F	TGCTGAACCACCTTTGATCTATTCTG	Forward	Splice variants
ER-ex8-R (R2)	CTCTGCTTCCGGGGGATGTAG	Reverse	Splice variants and constitutive
ER-ex8int-R	GATGTAGTAGGTTTGTAAAGGAATGTGCTG	Reverse	Splice variants
ERCsv1-F(F1)	CTGGCCTACAGATTTTGCTTGTCTCTC	Forward	rERCsv1 and1-2
ERCsv2-F (F3)	CCTTTGACTAAGTGCCCTTTTCCAG	Forward	rERCsv2 and1-2
ERCsv1-2-F(F2)	GTCTCTCTCGATTCTCGGAAAGAACCT	Forward	rERCsv1-2
ER-ex7_8-F (F4)	TTCCCATATCCGGCACATGAGTAACA	Forward	Constitutive
ER-ex5_6-F (F5)	CTCCTAACTTGCTCTTGGACAGGAATC	Forward	Insertless
ER-ex6-R (R1)	ATTGATTTGAGGCACAAACTCTTC	Reverse	Insertless and splice variants
rGAPD-F	GTCGTGGAGTCTACTGGTGTCTTC	Forward	GAPDH
rGAPD-R	TGGATGCAGGGATGATGTTCTGG	Reverse	GAPDH