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

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ABSTRACT

Estrogen receptor α (ERα) mediates estrogen diverse actions on tissues. ERα 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 ERα 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 six fold 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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1. Introduction

Estrogen receptor alpha (ERα) is one of the most studied transcription factors. ERα and its counterpart ERβ 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 ERα function and regulation of protein expression (Arnal et al., 2012).

The expression of ERα 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 ERα gene expression (Ishii et al., 2010; Kobayashi et al., 2011). At the post transcriptional level, several micro RNAs (miRNAs) target ERα and coregulators (Klinge, 2012). miR-22, miR-221, miR-222, miR-18a,b, miR-193, miR-302c, miR-206 downregulate the ERα gene. Human ERα has two miR-206 target sites in its 3'-UTR. This miRNA represses hERα 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 ERα gene expression. At the posttranslational level, estrogen binding to ERα 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 ERα transcriptional activity; S-nitrosylation inhibits ERα's ability to bind to estrogen response elements (EREs); and MAP kinase dependent phosphorylation is important for ligand independent activation (Cui

Abbreviations: ERα, estrogen receptor alpha; SRC, steroid receptor coactivator; WT, wild type; ERE, estrogen response element; rERCsv, rat estrogen receptor C-terminus splice variant.

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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 ERΔE3, ERΔE5, ERΔE7 and truncated ER product-1 (TERP-1, a unique 5'-untranslated region fused to exons 5–8 of ERα) have dominant negative effects on ERE containing promoters (Bollig and Miksicek, 2000; García Pedrero et al., 2003; Schreihofer et al., 1999). Certain variants also showed a positive effect on WT ERα function. At low concentration, TERP-1 stimulated ERα-mediated activation of a model promoter (Schreihofer et al., 1999). ERΔE3 variant activated a fragment of ovalbumin promoter having only ERE half site and AP-1 sites. These stimulatory effects of variants on ERα activity were attributed to their interaction with coregulators.

Although the ERα 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 ERα 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 ERα 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). Estrogen, 17-β-estradiol, was from Sigma. Estradiol [2,4,6,7,16,17-^{3}H(N)] (152 Ci/mmol), γ-^{32}P labeled ATP and poly dI-dC ● 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 μg/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 ERα'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 ERα 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) ERαs both WT and rERα C-terminus splice variants (rERCsvs) were PCR amplified using primers GCGC*AAGCTT*GCCACCATGACCATGACCCTTCACACC.

Table 1
Primers used in this study. All primers were designed based on the sequence of rat ERα gene (X61098) and rat GAPDH gene (XM_573217).

Name	Primer sequence	Orientation	Detection
ER-GSP-R	TCTCAGATGGTGTTGGGGAAG	Reverse	cDNA
ER-ATG-F	ATGACCATGACCCTTCACACC	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)	CTCTGCTTCCGGGGGGATGTAG	Reverse	Splice variants and constitutive
ER-ex8int-R	GATGTAGTAGGTTTGTAAGGAATGTGCTG	Reverse	Splice variants
ERCsv1-F (F1)	CTGGCCTACAGATTTTGCTTGTCTCTC	Forward	rERCsv1 & 1 and 1–2–2
ERCsv2-F (F3)	CCTTTGACTAAGTGCCCCTTTTCCAG	Forward	rERCsv2 1 and 1–2
ERCsv1-2-F (F2)	GTCTCTCTCGATTTCTCGGAAAGAACCT	Forward	rERCsv1-2
ER-ex7_8-F (F4)	TTCCCATATCCGGCACATGAGTAACA	Forward	Constitutive
ER-ex5_6-F (F5)	CTCCTAACTTGCTCTTGGACAGGAATC	Forward	Insertless
ER-ex6-R (R1)	ATTGATTTGAGGCACACAAACTCTTC	Reverse	Insertless and splice variants
rGAPD-F	GTCGTGGAGTCTACTGGTGTCTTC	Forward	GAPDH
rGAPD-R	TGGATGCAGGGATGATGTTCTGG	Reverse	GAPDH

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