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Human C-terminally truncated ERα variants resulting from the use of alternative exons in the ligand-binding domain

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ABSTRACT

The nuclear receptor genes contain alternative internal and terminal exons, with alternative exon incorporation yielding mRNA variants that encode various receptor types, including some with C-terminal truncation that exhibit constitutive activation or dominant-negative transcriptional transactivation. However, C-terminally truncated estrogen receptor α (ERα) variants with alternative sequences have rarely been reported in humans. Therefore, we assessed human ERα genomic organization and alternative splicing profiles, and identified both alternative exons and C-terminally truncated ERα variants. These naturally occurring C-terminally truncated ERα proteins were localized in the nuclei of transfected cells. In addition, ERαi45c and ERαΔ5 variants exhibited constitutive transactivation of an estrogen responsive element-driven promoter in transfected cells. We manufactured expression vectors encoding artificially truncated ERα constructs and evaluated their transactivation abilities to establish mechanisms determining the constitutive activity and dominant-negative properties of truncated variants. Lack of the region encoded in exon 8 eliminated basal and ligand-induced transcriptional transactivation. The C-terminally truncated ERα variants/constructs containing the helices 5 in their ligand-binding domains did not exhibit constitutive transactivation. Furthermore, we demonstrated that truncation from C-termini to helices 5 in the variant ligand-binding domains was required for constitutive activation and found that the remnant regions of the ligand-binding domains and variant-specific sequences influenced transcriptional transactivation efficiency. In conclusion, we elucidated the structural and functional features of novel C-terminally truncated ERα variants and revealed the mechanisms underlying constitutive transactivation by C-terminally truncated nuclear receptor variants.

1. Introduction

Nuclear estrogen receptors (ERs) are the main mediators of estrogen signaling and have a wide range of physiological roles in both reproductive and non-reproductive systems (Couse and Korach, 1999; Nilsson and Gustafsson, 2010). They are also associated with multiple pathophysiological conditions, such as cancer, cardiovascular and cerebrovascular diseases, and osteoporosis (Koellhoffer and McCullough, 2013; Jia et al., 2015). ERs belong to the nuclear receptor superfamily and act as ligand-induced transcription factors. Humans express two forms of ERs: estrogen receptor α (ERα) and estrogen receptor β (ERβ), which are encoded on separate genes, *ESR1* and *ESR2*, respectively. Like other members of

Abbreviations: 3′-RACE, Rapid amplification of cDNA 3′-ends; AF-1, activation function-1; AF-2, activation function-2; CTERP, C-terminally truncated estrogen receptor α product; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; ERα, estrogen receptor α; ERα46, N-terminally truncated 46 kDa estrogen receptor α; ERα66, 66 kDa estrogen receptor α; ERαΔ5, Δexon 5 estrogen receptor α; ERαΔ7, Δexon 7 estrogen receptor α; ERαDup5, exon 5-duplicated estrogen receptor α; ERβ, estrogen receptor β; ERE, estrogen responsive element; FBS, fetal bovine serum; ORF, open reading frame; PBS, phosphate-buffered saline.

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the nuclear receptor superfamily, the genes consist of several 5′-untranslated exons and eight coding exons. Their translated products contain functionally distinct domains common for all nuclear receptors (Nilsson and Gustafsson, 2010): an N-terminal ligand-independent transactivation domain (activation function-1, AF-1) (encoded by exons 1 and 2), a DNA binding domain (exons 2–4), a hinge domain (exon 4), and a ligand-binding and C-terminal transactivation domain (activation function-2, AF-2) (exons 4–8). In general, the ligand-binding domain possesses a conserved structure with 12 helical motifs (helices 1–12) (Bourguet et al., 1995), and conformational changes in these helices are involved in ligand-dependent transactivation processes, such as ligand-binding, agonist/antagonist recognition, coactivator binding, and transcriptional transactivation (Brzozowski et al., 1997; Moras and Gronemeyer, 1998; Zhang et al., 2005). The ligand binding domain of ERα lacks helix 2 and contains 11 helices (helices 1, 3–12) (Tanenbaum et al., 1998; Ruff et al., 2000).

Nuclear receptor pre-mRNAs are subject to complicated alternative splicing, which contributes to the diverse heterogeneity of the resulting mRNAs and proteins, each with distinct structures and functions. Multiple ER splice variants have been documented, with most of them produced by alternative use of 5′-untranslated exons (Kos et al., 2001; Ishii et al., 2010; Ishii and Sakuma, 2011; Kobayashi et al., 2011; Ishii et al., 2013) and/or by not incorporating conventional coding exons (Hirata et al., 2003). Furthermore, the ER genes possess alternative terminal exons, and their alternative selection results in mRNAs encoding C-terminally truncated ER variants. Two pioneer studies by Ogawa et al. (Ogawa et al., 1998) and Moore et al. (Moore et al., 1998) have reported the presence of human C-terminally truncated ERβ variants (ERβ2/βcx, ERβ3, ERβ4, and ERβ5) that are generated by alternative incorporation of alternative terminal exons located 3′-downstream of the conventional coding exon 8. The C-terminally truncated ERβ variants have dominant-negative effects on ERα- and/or ERβ-mediated transactivation (Ogawa et al., 1998; Poola et al., 2002). Moreover, expression of ERβ variants has been associated with several pathophysiological conditions (Leygue et al., 1999; Fujimura et al., 2001; Tong et al., 2002; Li et al., 2013; Liu et al., 2013; Chantzi et al., 2014; Huang et al., 2014; Wimberly et al., 2014). In the case of human ERα variants, a broad range of C-terminally truncated ERα variants have been reported to result from exon skipping and/or truncation of coding exons, and transcriptional frame shifts (Daffada and Dowsett, 1995; Murphy et al., 1996; van Dijk et al., 2000; Poola and Speirs, 2001; Perlman et al., 2005; Weickert et al., 2008; Ishunina and Swaab, 2012). The Δexon 5 ERα (ERαΔ5) variant is one of single exon-skipping variants resulting in C-terminal truncation (Fuqua et al., 1991; Bollig and Miksicek, 2000). Although there are several conflicting reports on the functions of ERαΔ5 (Fuqua et al., 1991; Castles et al., 1993; Ohlsson et al., 1998; Bollig and Miksicek, 2000), initial studies (Fuqua et al., 1991; Castles et al., 1993) demonstrated constitutive transcriptional transactivation of an estrogen responsive element (ERE)-driven promoter by the variant. Furthermore, its elevated expression was suggested to be involved in the progression of prostate adenocarcinoma (Taylor et al., 2010b). We recently identified multiple C-terminally truncated ERα variants with unique nucleotide sequences in mice (Ishii et al., 2011). These variants are produced by splicing to alternative terminal sequences localized between exons 4 and 5, and their products mediate constitutive transcriptional transactivation of an ERE-containing promoter in transfected HEK293 cells. These results indicate the potential presence of novel terminal exons in the human ERα gene associated with the production of C-terminally truncated ERα variants with distinct sequences and functions. However, with the exceptions of ERα36 (Wang et al., 2005; Wang and Yin, 2015), 113 bp-inserted ERα (Weickert et al., 2008), and ERα Δ4 + 49 bp (Ishunina and Swaab, 2012) variants, information on human C-terminally truncated ERα transcripts containing unique alternative sequences are limited. Therefore, we decided to examine the genomic organization of the human ERα gene and analyze splicing profiles of ERα mRNAs.

In the present study, we cloned C-terminally truncated ERα variants generated by utilization of alternative sequences in humans and characterized their fundamental transcriptional transactivation properties in transfected cells. Furthermore, we determined the mechanisms underlying constitutive transcriptional transactivation by the variants, focusing on helical structures in the ERα ligand-binding domain.

2. Materials and methods

2.1. Total RNA

Total RNAs from human normal organs were purchased from TaKaRa-Clontech (Shiga, Japan) and Life Technologies (Carlsbad, CA, USA). Information on the total RNAs is provided in Table S1.

MCF-7 cells were cultured as described elsewhere (Kobayashi et al., 2011), and their total RNA was isolated using RNAiso *Plus* (TaKaRa-Clontech) according to the manufacturer's instructions. The total RNA was treated with TurboDNase (Life Technologies) and re-purified.

2.2. Rapid amplification of cDNA 3′-end (3′-RACE) and RT-PCR

3′-RACE was performed as described previously (Ishii et al., 2009). Total RNAs extracted from the uterus and MCF-7 cells were reverse-transcribed with adaptor-Oligo(dT) primers, and 3′-RACE products were amplified by using nested PCR with forward primers designed against human ERα exons 4–7 and universal reverse primers.

RT-PCR was performed as described by Kobayashi et al. (Kobayashi et al., 2011) and Ishii et al. (Ishii et al., 2010) with some modifications. Total RNAs were reverse-transcribed with oligo(dT) primers. For expression analysis, cDNA (100 ng or 25 ng/tube) was PCR-amplified in three steps using Blend Taq polymerase (Toyobo, Osaka, Japan). For open reading frame (ORF) cloning, nested PCR was conducted using LA Taq polymerase (TaKaRa-Clontech).

Amplified products were separated on agarose gels by electrophoresis and stained with ethidium bromide. Gel images were obtained under UV irradiation using an ASTEC Gel Scene System (ASTEC, Fukuoka, Japan). 3′-RACE and RT-PCR amplicons were cloned into pGEM-T Easy (Promega, Madison, WI, USA) and then sequenced.

The oligonucleotide primers used for 3′-RACE and RT-PCR were purchased from Nihon Gene Research Laboratories (Sendai, Japan). Information about these primers is presented in Table S2.

2.3. Plasmid vectors

The ORFs of wild-type and C-terminally truncated ERα variants were introduced into pcDNA3.1(+)/Hygro or pcDNA3.1(−)/Hygro expression vectors (Life Technologies). The fragments of mouse ERα variants were PCR-amplified from pcDNA3.1/Hygro-mouse ERα66 vectors constructed in our previous study (Ishii et al., 2011). The coding region of human p300 was cloned into the pCMV-Tag2A expression vector (Agilent Technologies, Santa Clara, CA, USA) and fused in-frame with a FLAG epitope tag sequence. The cloned fragments were DNA sequenced. Firefly luciferase reporter vectors (pERE-Luc and pControl-Luc) and a *Renilla* luciferase reporter vector (pRL-TK) were obtained from Affymetrix (Santa Clara, CA, USA) and Promega, respectively. pERE-Luc contains three tandem