Down but not out? A novel protein isoform of the estrogen receptor α is expressed in the estrogen receptor α knockout mouse

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Abstract

The mouse knockout of the estrogen receptor α (ERα) gene, known as αERKO, has been extensively used for several years to study the role and function of ERα. Residual estradiol binding capacity in uterine tissue of 5–10% raised doubts if this knockout is a genuine null mutation of ERα. Although alternatively spliced ERα mRNA variants in the αERKO mouse were reported previously, the corresponding protein isoforms have not been detected to date. Here we show that a variant ERα protein, 61 kDa in size, is expressed in the uterine tissue of αERKO mice as a result of an alternative splicing. The transactivation capability of this protein is cell dependent and can be as high as 75% of the wild type ERα.

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Introduction

Estrogens play an important role in the development and physiological processes in vertebrates. Although they are primarily known as female sex hormones controlling female sex determination, reproduction and pregnancy, they are also involved in the development and maintenance of male reproductive organs (Hess et al. 1997) and in other physiological processes involving the liver, fat, bone metabolism and cardiovascular and neuronal activity (Norman & Litwack 1987, George & Wilson 1988, Auchus & Fuqua 1994). The role of estrogens in several pathological processes such as osteoporosis (Horowitz 1993), breast and endometrial cancers (Henderson et al. 1988), arteriosclerosis and Alzheimer’s disease (Auchus & Fuqua 1994) is also well established.

The generation of the estrogen receptor α (ERα) knockout mouse (αERKO) (Lubahn et al. 1993) was highly informative towards understanding the mechanisms of estrogen action in the complexity of the whole organism. An impressive and continuously growing body of information on ERα functions has been obtained from αERKO mice (Couse & Korach 1999 and references therein). However, the residual estradiol binding activity in uterine tissue in conjunction with the fact that the ERα gene was disrupted by an insertion of the neomycin gene into the first coding exon raised the possibility that alternatively spliced ERα variant(s) might be present in the αERKO mice. Two alternatively spliced transcripts of the disrupted ERα gene, called E1 and E2, were described in αERKO mice (Couse et al. 1995). The E2 mRNA contains a frame shift generating several stop codons after the first 41 amino acids of ERα. The E1 mRNA is generated by an alternative splicing, which utilizes a cryptic donor splice site at the beginning of the neomycin gene and the endogenous acceptor splice site of coding exon 2 (Fig. 1). The ERα protein isoform resulting from this transcript would have amino acids 92 to 155 of wild type ERα replaced by seven amino acids from the neomycin insert. This replacement would affect AF-1 transactivation function but not the DNA or ligand binding domain, which contains the AF-2 transactivation function. Such a protein isoform has recently been observed in mouse tissues (Pendaries et al. 2002). The discovery of estrogen
receptor β (ERβ) (Kuiper et al. 1996) brought another potential explanation of the observed binding activity. However, overlapping but distinct phenotypes of ERβ knockout (Krege et al. 1998) and ERFβ double knockout (Couse et al. 1999) mice do not fully support this explanation. More recently, ERFα, ERβ and ERFβ knockout mice were generated by complete deletion of the second coding exon using loxP sites and crosses with CMV-Cre mice (Dupont et al. 2000). These new knockout mice share many phenotypes with the original ER knockouts.

In this report, the presence of an ERα protein isoform, which might correspond to a putative translational product of the E1 transcript isolated previously by Couse et al. (1995) in αERKO uterine tissue is shown. The capability of this E1 isoform to activate a promoter containing an estrogen response element (ERE) was evaluated by transient transfections in different cell lines.

**Materials and methods**

**Plasmids and constructs**

The mouse ERα expressing vector pSG5-mERα was constructed by subcloning the ERα coding sequence from pMT2-MOR (kindly provided by M G Parker) into the EcoRI site of the pSG5 vector (Green et al. 1988). To create the pSG5-E1 vector expressing the E1 ERα, the ERα coding sequences upstream and downstream of the deletion were PCR amplified from the pSG5-mERα plasmid using the following primer pairs: PF1 (5’-TCGAAA TTGATGACCATGACCCCTGACACAAAGC-3’) and PR1 (5’-ATTAGACCGGTAGAATTCTCTAGCAAGCGGGCCGCCTCCGACCCGGGCG TAG-3’); and PF2 (5’-GCTTGCAGGAATTCTA CGGGGTCTTAATTCTGACAATCGACGCCAG AATGAGGCCGAG-3’) and PR2 (5’-GCGAATTC GGGGAGCCTGGGAGCTCTCAGAT-3’). The PF1 spans the translation start site of ERα and contains a BamHI site at its 5’ end. The PR2 primer spans the termination codon of ERα and contains an EcoRI site. The PR1 and PR2 primers are partially overlapping and contain sequences spanning the deletion. The two amplified products were isolated from the gel and were used as templates in a new PCR amplification using, again, primers PF1 and PR2. The final PCR product was directionally cloned into the BamHI and EcoRI sites of the pSG5 vector. The construct was sequenced.

The reporter plasmid ERE-tk-Luc containing the estrogen response element upstream of the thymidine kinase (tk) promoter and firefly luciferase encoding sequence was kindly provided by P Webb and has been described (Paech et al. 1997) previously. The pSG5-Renilla vector was kindly provided by M Hentze and contains the Renilla luciferase coding sequence cloned between the SmaI and BamHI sites of pSG5.

**Protein isolation and Western blot analysis**

The uteri were powdered in liquid nitrogen using a pestle and mortar. The powdered tissue was transferred into approximately 5 volumes of the buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and the protease inhibitor cocktail Complete mini (Roche). After 10 min incubation on ice, the suspension was pelleted in a table minicentrifuge and the supernatant was transferred into a new tube. The protein concentration was determined by the Bradford assay (BioRad, Munich, Germany). The protein extracts were stored at −70°C. Aliquots of 50 µg total proteins were analyzed by Western blotting using the following antibodies: rabbit polyclonal antibody MC-20 (SantaCruz, Heidelberg, Germany) raised against the last 20 amino acids of mouse ERα; rabbit polyclonal antibody H-184 (SantaCruz) raised against the 2–185 amino acids of human ERα; and rat monoclonal H222 antibody raised against the ligand binding domain of human ERα (Greene et al. 1984). The H222 antibody was kindly provided by G L Greene (University of Illinois, Urbana, IL, USA). The rabbit polyclonal anti-β-actin antibody (Sigma) was used to control for equal loading and degradation. The secondary horseradish peroxidase conjugates were purchased from DiaNova (Hamburg, Germany).

**Cell culture, transient transfections and dual luciferase assays**

HeLa, Ishikawa and NIH-3T3 cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (100 U/ml; Invitrogen), streptomycin...
(100 µg/ml; Invitrogen) and glutamin (2 mM, Invitrogen) at 37 °C in a 5% CO2 incubator. The cells were split 48 h before transfection into 24-well plates and the next day were transferred to the medium without phenol red and supplemented with 2.5% charcoal stripped FBS. Transfections were performed using Fugene6 reagent (Roche) following the recommendations of the manufacturer. In each transfection, 200 ng ERE-tk-Luc reporter construct, 50 ng of either pSG5-mERα or pSG5-E1 vector and 5 ng pSG5-Renilla vector were used. Immediately after transfection the vehicle, 17β-estradiol (10⁻⁸ M) or ICI 182,780 (10⁻⁸ M) were added to the media. Twenty hours after transfection cells were lysed in passive lysis buffer (Promega) and dual luciferase assays (Promega) were performed following the recommendations of the manufacturer. Ten microliters of cell lysate were transferred into a 96-well plate and the light emission was measured for 10 s with 2 s delay after the injection of 50 µl luciferase assay reagent or Stop&Glow reagent on an EG&G Berthold Microplate Luminometer LB 96V (Berthold Technologies, Bad Wildbad, Germany). All experiments were performed at least three times in triplicate.

Results

A novel ERα protein isoform is expressed in αERKO uteri

In order to investigate if ERα protein isoform(s) are expressed in αERKO mice, uterine samples from several animals of different ages were analyzed by Western blotting. A protein of approximately 61 kDa was detected by the MC-20 antibody that recognizes the last 20 amino acids of the mouse ERα receptor protein (Fig. 1A). This antibody produces an unspecific doublet at the 55 kDa range. The other proteins of approximately 52, 40 and 37 kDa are degradation products of ERα, as they can be partially prevented by inclusion of iodoacetamide or molybdate in the extraction buffer (Horigome et al. 1987 and our observation). Nevertheless, the presence of the same degradation product...
pattern in the αERKO samples indicates that the observed 61 kDa protein is likely to be an ERα isoform. The same protein was also detected by the H-184 antibody specific to the N-terminus of ERα (Fig. 1B). However, the H222 monoclonal antibody, which is specific to the ligand binding domain of the human ERα but cross-reacts with murine ERα (Greene et al. 1984, Horigome et al. 1987) failed to detect any ERα protein in αERKO animals by Western blots (data not shown). Although a ligand binding peak from αERKO uterine tissue analyzed by sucrose gradient centrifugation was shifted by H222 (Couse et al. 1995), a small fraction of animals did not express this protein (αERKO sample 2 in Fig. 1A). In total, uterine tissue from 13 animals was analyzed and, with the exception of two cases, the expression of this 61 kDa ERα protein was detected. The levels of expression estimated from Western blots with H-184 and anti-β-actin antibodies (Fig. 1B) varied between animals.

The size and the fact that the 61 kDa protein was detected by both N-terminal-specific and C-terminal-specific anti-ERα antibodies indicate that this protein isoform might be a putative translation product of the E1 ERα transcript observed previously in αERKO uteri (Couse et al. 1995). The transactivation capability of E1 ERα was previously characterized in COS-1 cells to be 25–30% of the wild type level (Couse et al. 1995). However, as ERα acts in COS-1 cells mainly through the AF-1 (El Tanani & Green 1997) we evaluated the capability of the E1 ERα variant to trans activate a promoter containing an estrogen response element by transient transfections in other cell lines: HeLa, NIH-3T3 and an ERα negative variant of the Ishikawa cell lines (Fig. 2A). The induction of the luciferase reporter gene expression by E1 ERα compared with wild type ERα varied between 30% (Ishikawa cells) and 75% (HeLa cells). The activity was completely abolished by antiestrogen ICI 182,780 (ICI). The levels of expression of both E1 and wild type ERα proteins in transiently transfected HeLa cells were comparable (Fig. 2B). Both protein isoforms were also down-regulated by estradiol and ICI treatment as previously reported (Read et al. 1989, Gyling et al. 1990, Gibson et al. 1991, Dauvois et al. 1992, Wijayaratne & McDonnell 2001) (Fig. 2B).

In summary, these results indicate that a novel ERα protein isoform is expressed in the majority of αERKO animals. This protein might correspond to the putative E1 ERα described previously. The activity of the E1 ERα variant is cell dependent and would have the capability to significantly contribute to ERα signaling in certain cell types or tissues.

Discussion

When the αERKO mouse was created the current technology utilized at the time was the insertional disruption of genes. The 1·8 kbp neomycin encoding sequence was inserted into the NotI site of the first coding exon of the murine ERα gene (Lubahn et al. 1993). As all the coding exons of the ERα gene are still present in the genome of αERKO mouse, the possibility of alternative splicing that generates mRNA variants encoding truncated or deletion versions of ERα protein exists. Such alternative splicing was observed, for example, in a knockout mouse in which the
transforming growth factor α gene was disrupted by insertion (Luetteke et al. 1993). Similarly, alternatively spliced ERα mRNAs, named E1 and E2, were also observed in αERKO mice (Couse et al. 1995). While the E2 mRNA encodes only the first 41 amino acids of ERα, the E1 mRNA encodes a potential ERα isoform with a deletion in the B domain containing the AF-1 function. However, the corresponding E1 ERα protein has been detected in αERKO tissues only recently (Pendaries et al. 2002). The fact that the H222 antibody was originally used to analyze the presence of ERα protein isoforms in αERKO uterine tissue by Western blot techniques (Couse et al. 1995) might explain the failure to detect the E1 ERα isoform which is expressed in αERKO at lower levels than ERα in wild type mice. In fact, the mRNA for the E1 variant was only detected by RT-PCR techniques. General experience suggests that although the H222 antibody readily interacts with ERα from various species (Greene et al. 1984) in native conditions, such as in sucrose gradients where E1 binding was demonstrated, it may not perform well under denaturing conditions, such as Western blot involving low amounts of protein. In contrast, the rabbit polyclonal antibodies used in this study readily detect ERα in either murine or human tissues in Western blots, therefore allowing the detection of E1 ERα in αERKO uteri.

The penetrance of the E1 variant expression differed between animals and varied from undetectable (in two of thirteen animals tested in total) to varying levels in most of the animals. The influence of genetic background or generation number on the penetrance of a mutant phenotype has been documented in several cases and might also account for the observed differences in E1 ERα expression in αERKO (Dunn et al. 1997, Herrera et al. 1999, Wawersik et al. 1999, Kume et al. 2000, Nadeau 2001). Nevertheless, incomplete penetrance might be an advantage, as approximately 15% of αERKO animals seem to represent genuine ERα-null mutants while the rest can be regarded as AF-2 hypomorphic AF-1 knockouts. Thus the αERKO mouse might represent a good model system to investigate the comparative physiological roles of AF-1 and AF-2 in ERα function.

The dramatic difference in the activity of E1 ERα obtained in transfection experiments using various cell lines is not surprising, as AF-1 and AF-2 activities are cell type and promoter dependent (Tora et al. 1989, Berry et al. 1990, Metzger et al. 1995, Flouriot et al. 2000). As the E1 ERα has impaired AF-1 function but conserved AF-2 function, it can be anticipated that only small differences in transactivation capabilities between E1 and wild type ERα would be observed in cells with strong AF-2 context such as HeLa (Tora et al. 1989). The cell context most likely depends on the blend of AF-1 or AF-2 specific coactivators and/or corepressors present in the cell. Therefore, it is difficult to predict the activity of E1 ERα in various cell types and especially in tissues. The high similarity in the reproductive and general phenotypes of αERKO and the recently generated ERα knockout mice (Dupont et al. 2000) might indicate that E1 ERα plays only a minor role in the physiology of the reproductive tract in αERKO mice. Interestingly, use of the αERKO mice has shown a lack of ligand independent growth factor signaling in vivo with either epidermal growth factor (Curtis et al. 1996) or insulin-like growth factor-I (Klotz et al. 2002), which would be a clear demonstration that ERα AF-1 contributes to the cross talk signaling in vivo.

To conclude, our demonstration that a novel ERα protein isoform is expressed in the αERKO mouse indicates that data obtained from studies using αERKO mice should be further considered with respect to AF-1 and AF-2 functions. However, the variable penetrance of this phenotype in αERKO animals means that ERα isoform status can serve as a model to study the AF-1 and AF-2 functions within individual tissues in the mouse.

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