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The Naturally Occurring Variant of Estrogen Receptor (ER) ERΔE7 Suppresses Estrogen-Dependent Transcriptional Activation by Both Wild-Type ERα and ERβ

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We have isolated and functionally characterized the exon 7-skipped variant (ERΔE7) of estrogen receptor (ER)α, which has emerged as the predominant variant expressed in multiple normal and tumoral tissues. However, to date no function has been established for this variant in mammalian cells. ERΔE7 exhibits a negligible ability to bind ligands, insensitivity to allosteric modulation by estrogen and antiestrogens, and loss of estrogen-dependent interaction with p160 coactivators such as SRC-1 and AIB1. ERΔE7 is able to form heterodimers with both ERα and ERβ in a ligand-independent manner. Transient expression experiments in HeLa cells show that increasing amounts of ERΔE7 result in a progressive inhibition of the estrogen-dependent transcriptional activation by both wild-type ERα and ERβ on estrogen response element-driven promoters. The inhibitory effect of ERΔE7 is due to the inhibition of binding of wild-type receptors to their responsive elements. Surprisingly, the activation function (AF)-1-dependent transactivation triggered by epithelial growth factor and phorbol-12-myristate-13-acetate is also abolished in ERΔE7 despite AF1 integrity, suggesting a cross-talk between AF1 and AF2 regions of the receptor. These results indicate that the naturally occurring variant ERΔE7 is a dominant negative receptor that, when expressed at high levels relative to wild-type ERs, might have profound effects on several estrogen-dependent functions. (*Endocrinology* 144: 2967–2976, 2003)

ESTROGEN (E_2) has long been known to promote the growth of certain human neoplasms, notably tumors of the breast, endometrium, and pituitary. It also modulates the development and function of normal tissues, such as the mammary gland and bone. It also has influence on the cardiovascular and central nervous systems (1, 2). The mitogenic and regulatory effects of E_2 are mediated through two closely related nuclear receptors, estrogen receptor (ER)-α and the more recently described ERβ (3, 4), which are encoded by separate genes. Both receptors are members of the steroid receptor superfamily that act principally as ligand-activated DNA-binding dimers (5, 6). It is well documented the existence of multiple ERα variants generated by alternative splicing (exon skipping) of the single ERα pre-mRNA (7–9).

ERα is a protein composed of discrete functional domains. The DNA-binding domain consists of exons 2 and 3, each of which encodes a single zinc-finger motif. This domain is essential for sequence-specific DNA binding and transcriptional activation through canonical estrogen response elements (EREs) (10). The N-terminal transactivation function (AF)1 encoded by exon 1 and a portion of exon 2 operates in a ligand-independent manner and may be activated by a variety of agents (11, 12). A ligand-binding domain (LBD) confers regulatory function to the receptor and is encoded by exons 4–8. This region is the most complex functionally and includes determinants for 1) heat-shock protein association in the cytoplasm, 2) ligand-dependent receptor dimerization, 3) a ligand-dependent activation function (AF2), which promotes gene transcription by recruiting coactivators on ligand binding, and 4) estrogen and antiestrogen ligand binding (13–15). Both AF1 and AF2 domains are required for optimal stimulation of transcription, but their relative contribution varies in a promoter- and cell type-specific manner (16, 17).

Evidences for the function of ERα variants have been elusive. Thus, it has been reported that ERΔE5 can support weak, cell type-dependent activity (18, 19). Alternatively, both ERΔE5 and ERΔE3 have been reported as dominant negative receptor forms in the presence of wild-type (wt) ERα (20–22). With regard to ERΔE7, contrasting results have been obtained. Thus, it has been reported to repress 60% of the action of equimolar wt ERα in yeast (23, 24) and be ineffective as a dominant negative of ERα in mammalian cells (20, 21). Moreover, no studies exist in literature assessing the possible role of ERΔE7 on transactivation mediated by the more recently described ERβ.

This work focuses on the functional characterization of the exon 7-skipped variant of ERα (ERΔE7) isolated from MCF-7 cells. This is the most abundant splicing form of ERα expressed in this ER (+) mammary carcinoma cell line. We have examined the ability of the ERΔE7 to bind ligand, the interaction with coactivators, its heterodimerization with

Abbreviations: AF, Activation function; DTT, dithiothreitol; E_2, estrogen; EGF, epithelial growth factor; ER, estrogen receptor; ERE, estrogen response element; FCS, fetal calf serum; GST, glutathione-*S*-transferase; h, human; HA, hemagglutinin; LBD, ligand-binding domain; OHT, 4-hydroxytamoxifen; PMA, phorbol-12-myristate-13-acetate; wt, wild-type.

both wt ERα and ERβ, and its role in complex formation with DNA as well as on AF1- and AF2-dependent transcriptional activation. Our results indicate that ERΔE7 acts as a dominant negative receptor with ability to suppress the E_2-dependent transcriptional activation by both wt ERα and ERβ. Therefore, ERΔE7 that had been labeled as transcriptionally inert should be really considered an important receptor isoform in controlling E_2-dependent functions.

Materials and Methods

Materials

17β-Estradiol, 4-hydroxytamoxifen, epidermal growth factor, and other chemicals were purchased from Sigma (St. Louis, MO). ICI 182,780 was provided by Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Macckesfield, Cheshire, UK). [^{35}S]-methionine (Pro-mix; 14.3 mCi/ml; >1000 Ci/mmol) was from Amersham Pharmacia Biotech (Little Chalfont, UK).

Plasmids

Recombinant plasmids allowing expression of chimeric proteins containing ER sequences were constructed as follows. The cDNA fragments encoding LBD regions (exons 4–8) of the wt human (h) ERα and the exon 7-skipped variant were amplified by RT-PCR from MCF-7 cells using the following primers: 5′-CGGGATCCGGGTCTGCTGGAGAC-3′ (at positions 1133–1147) and 5′-GCGAATTCTCAGACTGTGGCAGGG-3′ (at positions 2065–2082). The amplification products of 964 and 780 bp were subcloned downstream of the glutathione-*S*-transferase (GST) gene into the *Bam*HI/*Eco*RI sites of pGEX-2TK vector (Amersham Pharmacia Biotech) to generate the recombinant plasmids pGTK-LBD and pGTK-LBDΔE7, respectively. Both constructions were verified to be free of mutations and in frame with GST by sequencing.

The expression vector pcDNA-ERα was constructed by ligating the full-length hERα cDNA into the *Bam*HI/*Eco*RI sites of the eukaryotic expression vector pcDNA 3 (Invitrogen, San Diego, CA), as we previously described (25). For pcDNA-ERΔE7 construction, the 518-bp *Bgl*II/*Eco*RI portion of the wt ERα from pcDNA-ERα plasmid was replaced with the corresponding exon 7-deleted ER fragment of 334 bp from pGTK-LBDΔE7 plasmid.

Expression and purification of recombinant proteins

The resulting GST fusion proteins were expressed in *Escherichia coli* and purified by adsorption onto glutathione sepharose essentially as described by Frangioni and Neel (26). SDS-PAGE analysis showed a molecular mass of about 65 kDa for GST-LBD (which contains residues 280–595 of wt hERα) and about 50 kDa for the truncated protein GST-LBDΔE7 (residues 280–466). The precise deletion of exon 7 results in a reading frame shift, causing premature termination of translation immediately downstream of the novel splice junction with the inclusion of 10 non-ER residues after codon 457 (23). Both wt and variant hybrid proteins were expressed at similar levels in *E. coli* as monitored by Coomasie-stained SDS-PAGE analysis.

Cell culture, transient transfections, and luciferase assay

HeLa cells were propagated as we previously described (27). Before transfection, HeLa cells were seeded in 12-well plates Linbro (ICN Biomedicals, Inc., Aurora, OH) and incubated 12–18 h at 37 C. Then cells were transferred to phenol-red free DMEM containing 0.5% charcoal/dextran-treated fetal calf serum (sFCS) and maintained for 3 d at 60–80% confluency. Cells were transfected with 0.5 μg of an ERE-driven reporter plasmid, 0.05–1.5 μg ER expression vectors and 50 ng of an internal control *Renilla* luciferase plasmid, pRL-TK (Promega Corp., Madison, WI) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's protocols. After 18–24 h, the medium was renewed and cells were stimulated during 24 h with different chemicals, as indicated.

Luciferase assays were performed as recommended by dual luciferase system (Promega Corp.). To correct for differences in transfection efficiency, the experimental values were normalized to *Renilla* luciferase activity.

To generate [Δ7ERα]MCF-7 cell lines, MCF-7 cells plated in a 10-cm culture dish (80% confluency) were stably transfected with 10 μg ERΔE7 expression vector using 75 μl lipofectamine and 50 μl Plus reagent (Invitrogen) following the manufacturer's instructions. After 24 h, medium was replaced with RPMI 1640 medium (BioWhittaker, Inc., Walkersville, MD) containing 10% heat-inactivated FCS and 500 μg/ml geneticin G418 (Invitrogen) for selection. The medium was renewed every 3–4 d. In 4 wk, visible colony foci were isolated and propagated in medium containing G418.

EMSA

Binding of the E_2-ER complex to ERE was performed as we previously described (27). Five to ten microliters of cellular lysates of transient transfections were mixed with buffer B (20 mM HEPES-KOH, pH 7.9; 10 mM $MgCl_2$; 1 mM EDTA; 10% (vol/vol) glycerol; 100 mM KCl; 0.2 mM phenylmethylsulfonyl fluoride; 0.2 mM dithiothreitol (DTT); 0.5% Nonidet P-40; and protease inhibitors) and incubated with 1 μg poly (deoxyinosine-deoxycytidine) in a total volume of 40 μl. Mixtures were preincubated at 0 C for 15 min, followed by incubation with the indicated hormones at 0 C for 10 min. [^{32}P]-labeled probe (10 fmol containing $3–5 \times 10^4$ dpm) was added to the reaction and allowed to proceed for 1 h at 0 C, followed by 30 min at room temperature. The samples were loaded onto a preelectrophoresed (10 mA) 5% polyacrylamide gel (acrylamide to bisacrylamide ratio of 40:1) in TBE (45 mM Tris Borate, 1 mM EDTA) at 11 mV/cm. For specificity assays, 100-fold excess of unlabeled oligonucleotide was used as competitor before adding the probe to the binding reaction.

In vitro protein-protein interaction assays

GST pull-down experiments were performed as previously described by Cavailles *et al.* (28). [^{35}S]-labeled proteins of wt hERα, hERβ, ERΔE7, SRC-1a, or AIB1 coactivators were synthesized by *in vitro* transcription-translation (Promega Corp.) using pcDNA-ERα, pCXN2-hERβ (29), pcDNA-ERΔE7, pCR-SRC-1a, or pcDNA-3.1AIB1, respectively, as templates. The fusion proteins loaded on glutathione-sepharose beads (25 μl) were preincubated with 1-μM concentrations of ligands [E_2, 4-hydroxytamoxifen (OHT), or ICI] for 30 min at 4 C, followed by incubation with [^{35}S]-labeled proteins for 1.5 h at 4 C in a total volume of 150 μl IPAB buffer [20 mM HEPES-KOH, pH 7.9; 5 mM $MgCl_2$; 150 mM KCl; 0.02 mg/ml BSA; 0.1% (vol/vol) Triton X-100; 0.1% Nonidet P-40; and protease inhibitors]. Beads were washed four to five times with IPAB without BSA, collected by centrifugation, and resuspended in 20 μl loading buffer for SDS-PAGE analysis. The gel was vacuum dried, and the radiolabeled products were visualized by autoradiography.

Far-Western blot experiments were carried out essentially as described by Cavailles *et al.* (28). Purified GST-proteins were subjected to SDS-PAGE and electroblotted onto nitrocellulose. After denaturation/renaturation in 6 M to 0.187 M guanidine hydrochloride in HB buffer (25 mM HEPES-KOH, pH 7.9; 25 mM NaCl; 5 mM $MgCl_2$; 1 mM DTT), filters were saturated at 4 C in blocking buffer and incubated with [^{32}P]-labeled GST-LBD probe (28) in H buffer (20 mM HEPES-KOH, pH 7.9; 75 mM KCl; 0.1 mM EDTA; 2.5 mM $MgCl_2$; 0.05% Nonidet P-40; 1% milk; 1 mM DTT) using 200,000 cpm of probe per milliliter in the presence of 1 μM E_2 and cold GST to block nonspecific binding. After washes with H buffer, filters were dried and exposed for autoradiography at −80 C.

Immunoprecipitation

Five microliters of *in vitro* translated [^{35}S]-labeled wt hERα or ERβ were mixed with equal amounts of [^{35}S]-labeled ERΔE7 posttranslationally and incubated with 30 μl buffer B [20 mM HEPES-KOH, pH 7.9; 10 mM $MgCl_2$; 1 mM EDTA; 10% (vol/vol) glycerol; 100 mM KCl; 0.2 mM phenylmethylsulfonyl fluoride; 0.2 mM DTT; 0.5% Nonidet P-40; and protease inhibitors] with the indicated hormones at 0 C for 10 min. Then ERα-ERΔE7 and ERβ-ERΔE7 heterodimers were immunoprecipitated with monoclonal anti-ERα antibodies NCL-ER-LH1 (Novocastra Laboratories, Newcastle upon Tyne, UK), C-314 (SC-786) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal anti-hemagglutinin (HA), or rabbit polyclonal anti-ER antibodies (raised in our laboratory against

C-terminal amino acid residues 280–595 of hERα) (25) on ice for 1 h, followed by incubation with 50 μl of 50% protein G sepharose slurry in buffer B at room temperature for 1 h by rocking. The immunoprecipitates were pelleted by centrifugation, washed, and analyzed by SDS-PAGE. After suitable separation, the gel was vacuum dried, quantified with an Instantimager (Packard, Downers Grove, IL) and exposed for autoradiography.

Western blot analysis

Western blot analysis was carried out as described (25) using monoclonal anti-ERα antibodies NCL-ER-6F11 (Novocastra Laboratories). Goat antimouse IgG antibodies coupled to horseradish peroxidase (Sigma) were used as secondary antibodies. Immunoreactive bands were visualized with the ECL detection system (Amersham Pharmacia Biotech).

Results

ERΔE7 binds neither estrogens nor antiestrogens

To identify and isolate ERΔE7, we performed RT-PCR assays using total RNA isolated from MCF-7 cells actively growing with 10% FCS and cells arrested at G_0-G_1 phase by FCS and estrogen depletion. Reverse transcription was carried out priming with oligo-dT. The synthesized cDNAs were then amplified by PCR using oligonucleotide primers that flank the LBD of wt hERα. Two forms of ERα (964 and 780 bp) were detected by hybridization with an internal probe, corresponding to wt ERα and ERΔE7, respectively (Fig. 1A). Thus, ERΔE7 appears as the predominant spliced variant of ERα in MCF-7 cells. This was confirmed when measurements of the protein levels were carried out (Fig. 1B). When reverse transcription reactions were performed by priming with a 3′-specific oligonucleotide complementary to sequences downstream of the termination codon of ERα ORF, additional PCR products corresponding to multiple ERα variants were detected, as previously described (30).

We investigated the ability of the LBDs of wt ERα and ERΔE7 to bind [^{3}H]-estradiol. Increasing amounts (0.75–3 pmol) of GST-LBD or GST-LBDΔE7 hybrid proteins purified and immobilized onto glutathione sepharose were incubated with [^{3}H]-estradiol and the hormone-receptor complex was determined by measuring the radioactivity retained on sepharose affinity matrices. Only wt ERα was able to bind [^{3}H]-estradiol, whereas ERΔE7 showed no specific ligand binding (Fig. 2, A and B).

Additional evidence for the lack of interaction of ERΔE7 with E_2 was obtained analyzing the specific effects of ligands

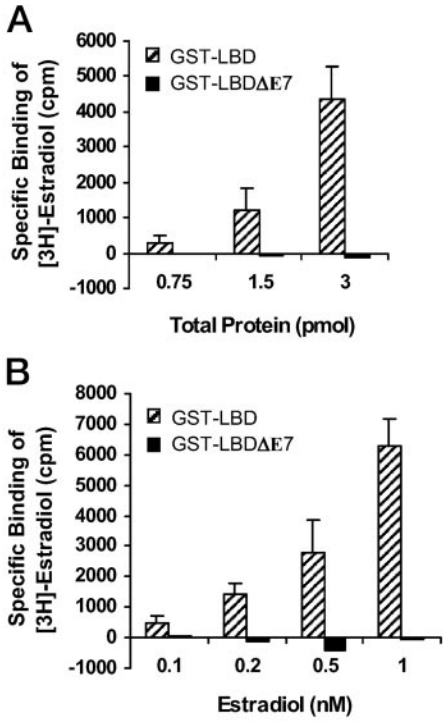

FIG. 2. Binding of [^{3}H]-estradiol by the fusion proteins containing LBD regions of the wt hERα and ERΔE7 variant. The fusion proteins were expressed in *E. coli* and purified by adsorption onto glutathione sepharose. A, Equal amounts of each protein (0.75–3 pmol, as indicated) were incubated with 10 nM [^{3}H]-estradiol or [^{3}H]-estradiol plus 1000-fold excess of unlabeled estradiol for 30 min at 4 C. The matrix was washed and pelleted to remove unbound ligand and the radioactivity determined. B, Binding to 10 pmol GST-proteins was determined varying the concentration of [^{3}H]-estradiol from 0.1 to 1 nM in the absence and presence of 1000-fold excess of unlabeled estradiol. Values correspond to specifically bound [^{3}H]-estradiol, and *bars* represent the mean ± SD of triplicates in two separate experiments.

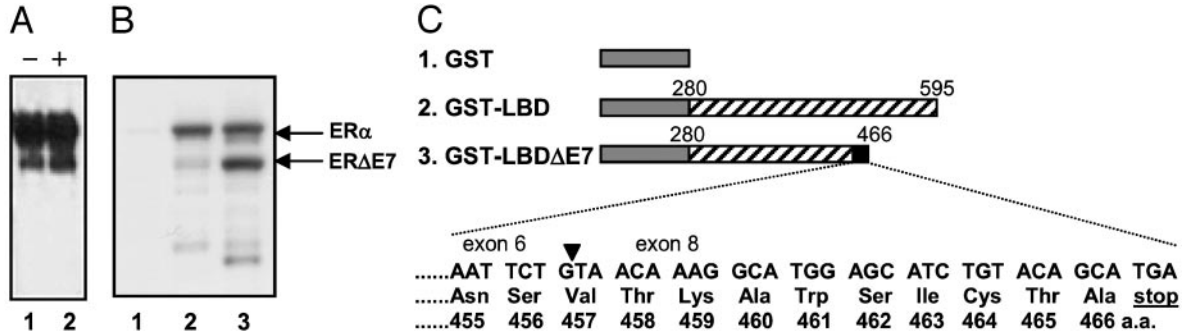

FIG. 1. ERΔE7 represents the most abundant of the ERα splicing variants in MCF-7 cells. A, Amplification of LBD region of ERα was performed by RT-PCR using total RNA extracted from proliferating MCF-7 cells (+) or G_0-G_1 arrested cells (−). PCR products were separated on agarose gels and hybridized with an internal 309-bp probe (positions 1454–1763). B, ERΔE7 protein expression was determined by Western blot analysis. Whole-protein extracts (100 μg) from HeLa (lane 1), MCF-7 (lane 2), and [Δ7ERα]MCF-7 cells (lane 3) were resolved on 10% SDS-PAGE and analyzed using the monoclonal anti-ERα antibody NCL-ER-6F11. C, Schematic description of GST fusion proteins, corresponding to GST fragment (lane 1), GST fused to the LBD of wtERα (residues 280–595) named as GST-LBD (lane 2), and GST fused to the LBD of ERΔE7 variant (residues 280–466) named as GST-LBDΔE7 (lane 3). The figure also includes sequence details of the exon 7 deletion, and *black triangle* points to the novel splice junction.

on receptor sensitivity to trypsin digestion, compared with that of wt ERα. This was accomplished by using *in vitro* [^{35}S]-labeled wt ERα and ERΔE7 and preincubating these receptors with E_2. The resulting complexes were then subjected to limited digestion with trypsin, and the products were analyzed by SDS-PAGE. The wtERα was highly sensitive to trypsin degradation in the absence of ligand (Fig. 3, lane 3). In the presence of E_2, however, a trypsin-resistant 32-kDa receptor fragment was observed (Fig. 3, lane 6). Incubation of the labeled receptor with the antiestrogens OHT or ICI also rendered a fragment that was resistant to further digestion with trypsin, as previously reported (31). ERΔE7 was also highly sensitive to protease digestion, but the presence of E_2 failed to protect this receptor from degradation by trypsin, as expected from its inability to bind ligand (Fig. 3, lanes 9 and 12). Moreover, neither OHT nor ICI protected ERΔE7 from digestion, indicating that ERΔE7 was also unable to interact with antiestrogens (data not shown). This suggests that this variant lacks allosteric modulation by both estrogen and antiestrogens. The deletion of exon 7 eliminates a significant portion of the LBD, and thus the loss of ligand binding should be expected.

ERΔE7 forms heterodimers with both wtERα and ERβ

We performed GST pull-down experiments with [^{35}S]-methionine-labeled ERΔE7 and GST-LBD of wtERα to assess the interaction between the two proteins *in vitro*. As shown in Fig. 4A, ERΔE7 protein was successfully coprecipitated with the GST-LBD fusion protein, and this interaction was unaffected by the absence (C) or presence of E_2 or OHT at 1 μM. This indicates that ERΔE7 heterodimerizes with wtERα in a ligand-independent manner.

To investigate dimer formation with ERΔE7, we used Far-Western blotting. This technique restricts the detection to direct interactions only between proteins. Thus, equal amounts of GST and the purified hybrid proteins GST-LBD and GST-LBDΔE7 (Fig. 4B, *right panel*) were immobilized onto nitrocellulose and, after denaturation/renaturation, proteins on filters were incubated with an *in vitro* [^{32}P]-labeled GST-LBD probe in the presence of 1 μM E_2 (Fig. 4B). The ERα probe was found to bind to both LBDs of wtERα and ERΔE7, whereas no interaction was detected with GST alone (Fig. 4B, *left panel*, lane 1). This experiment demonstrates the direct interaction between wtERα and ERΔE7 and reveals that the capacity of wtERα to heterodimerize with ERΔE7 variant appears to be comparable with its homodimerization ability.

Finally, we analyzed the interaction of ERΔE7 with both full-length wt hERα and ERβ, using in this case proteins in solution (Fig. 4C). For this purpose, aliquots of [^{35}S]-labeled wtERα or ERβ were mixed with an equal volume of [^{35}S]-labeled ERΔE7 and incubated in either the absence of hormone (C) or in the presence of 1 μM E_2 or 1 μM OHT, as indicated. ERα-ERΔE7 heterodimers formation was determined by immunoprecipitation with the monoclonal anti-ERα antibody NCL-ER-LH1, which recognizes an epitope located within the C-terminal of LBD (lanes 1–3). Because the truncated receptor ERΔE7 lacks this epitope, only ERα homodimers and heterodimers containing ERα and ERΔE7 will be immunoprecipitated by this antibody. Similarly, ERβ-ERΔE7 heterodimerization was detected by immunoprecipitation assays using the ERα-specific monoclonal antibody C-314 raised against the N-terminal of ERα that therefore allows visualization of both ERΔE7 homodimers and ERβ-ERΔE7 heterodimers (lanes 5–7). Additionally, total ERs were immunoprecipitated with polyclonal anti-ER antibodies as a control (lanes 4, 8, and 12). No immunoprecipitation was observed when an unrelated antibody (anti-HA) was used (lanes 9–11). These results imply that the ERΔE7 variant is able to form heterodimers with both wtERα and ERβ and these interactions are not subjected to hormonal regulation.

Coactivator-binding properties of ERΔE7

The LBD also includes a well-characterized C-terminal transactivation function (AF2), which promotes gene transcription by recruiting coactivator proteins in a ligand-dependent manner (32, 33). We tested whether exon 7 deletion might affect the binding of coactivators to ERΔE7. Thus, we determined the interaction of ERΔE7 with p160 coactivators *in vitro* by GST pull-down experiments using

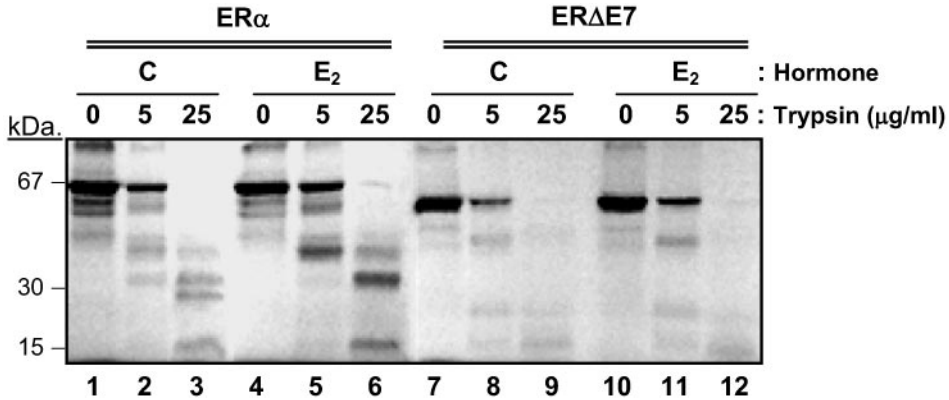

FIG. 3. Trypsin digestion of E_2-ligated and unligated wtERα and ERΔE7. Radiolabeled hERα and ERΔE7 were synthesized *in vitro* and subjected to digestion with different concentrations of trypsin (as indicated) in the absence or the presence of 1 μM E_2. The product of the digestion reactions were resolved by SDS-PAGE and visualized by autoradiography. Lanes 1–6 represent ERα digestions performed in the presence of ligand E_2 (lanes 4–6) or vehicle alone (ethanol) (C, lanes 1–3). Lanes 7–12 correspond to the products of identical reactions performed with ERΔE7 in absence (C, lanes 7–9) or presence of 1 μM E_2 (lanes 10–12).

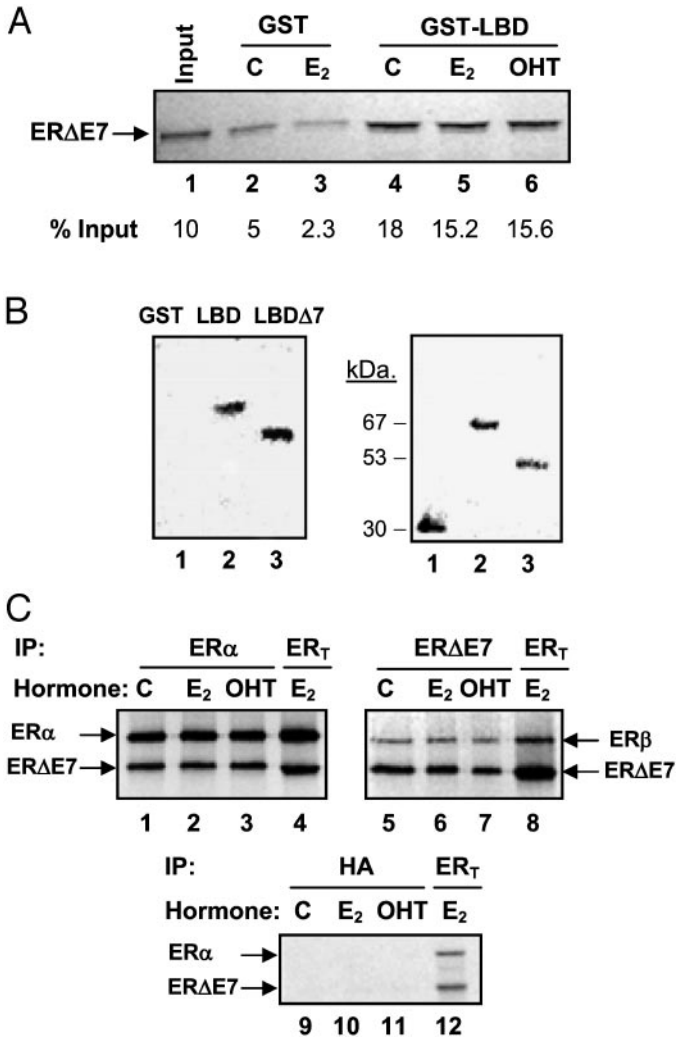

FIG. 4. ERΔE7 forms heterodimers with both wtERα and ERβ *in vitro.* A, GST pull-down experiment performed using *in vitro* translated [^{35}S]-methionine-labeled ERΔE7 incubated with GST alone (lanes 2 and 3) or GST fusion protein containing LBD of the wt hERα (GST-LBD, lanes 4–6) immobilized on GSH-sepharose in the absence of ligand (C) or presence of 1 μM E_2 or 1 μM OHT. *Below the panel*, the percentage of the input pulled down (counts per minute) is shown. The input lane represents 10% of the total amount of labeled ERΔE7 used in the binding reactions (lane 1). B (*left panel*), Far-Western analysis of GST fragment (lane 1), GST-LBD (lane 2), and GST-LBDΔE7 (lane 3) immobilized onto nitrocellulose and incubated with 200,000 cpm/ml [^{32}P]-labeled GST-LBD probe in the presence of 1 μM E_2. B (*right panel*), Coomasie-blue stained SDS-PAGE analysis of GST proteins used for Far-Western experiments. C, Equivalent aliquots of [^{35}S]-labeled wtERα and ERΔE7 were immunoprecipitated with the antibody NCL-ER-LH1 (which binds only to the wtERα) in the absence of ligand (C, lane 1) or presence of 1 μM E_2 (lane 2) or 1 μM OHT (lane 3). In parallel reactions, aliquots of [^{35}S]-labeled wtERβ and ERΔE7 were immunoprecipitated with the antibody C-314 (which binds only to the ERΔE7 variant) in the absence of ligand (C, lane 5) or presence of 1 μM E_2 (lane 6) or 1 μM OHT (lane 7). Lanes 4, 8, and 12 correspond to the immunoprecipitation of total ERs (ER_T) using polyclonal anti-ER antibodies, which bind wtERα, ERβ, and ERΔE7. Lanes 9–11 correspond to immunoprecipitation using anti-HA antibodies, as negative control. The lower level of ERβ in the immunoprecipitates is due to a low efficiency in the synthesis of this protein; nevertheless, ERβ fractions immunoprecipitated with C-314 antibody quantitatively represent about 50% of the total protein (compare lanes 5–7 with lane 8).

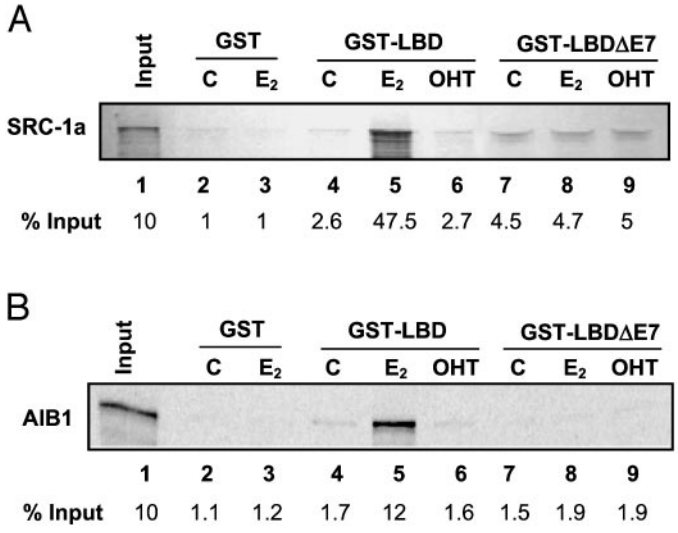

FIG. 5. Estrogen-dependent interaction of p160 coactivators with the AF2 domains of wtERα and ERΔE7. A, *In vitro* translated [^{35}S]-methionine-labeled SRC-1a was incubated with GST alone (lanes 2 and 3) or GST fusion proteins containing LBDs of the wtERα (GST-LBD, lanes 4–6) or the ERΔE7 variant (GST-LBDΔE7, lanes 7–9) immobilized on GSH-sepharose in the absence of ligand (C) or presence of 1 μM E_2 or 1 μM OHT. B, *In vitro* translated [^{35}S]-methionine-labeled AIB1 was treated as in A. *Below each panel*, the percentage of the input pulled down (counts per minute) for each assay is shown. In each panel, the input lane represents 10% of the total volume of lysate used in each reaction (lane 1).

[^{35}S]-labeled SRC-1a (Fig. 5A) or AIB1 (also named RAC3/ACTR/pCIP/SRC-3) (Fig. 5B) and GST-LBD or GST-LBDΔE7 hybrid proteins as affinity reagents. As expected, the binding of both p160 coactivators to LBD of wtERα was greatly stimulated in the presence of E_2, whereas the estrogenic antagonist OHT failed to induce their binding to the wtAF2 domain. On the other hand, using the GST-LBDΔE7 hybrid protein, no induction of coactivators binding was observed in the presence of either E_2 or OHT.

ERΔE7 inhibits E_2-dependent transcriptional activation by both wtERα and ERβ on ERE-driven promoters

To investigate the biological activity of ERΔE7, we constructed the expression plasmids pcDNA-ERα and pcDNA-ERΔE7 that contain the full-length cDNAs of wtERα and ERΔE7, respectively. Using *in vitro* transcription/translation systems, we determined that both constructs directed the expression of the corresponding [^{35}S]-labeled receptors with the expected sizes of 66 and 52 kDa (see Fig. 3, lanes 1 and 7). In agreement with previously published results (20, 23), we confirmed that ERΔE7 is an isoform of ERα that failed to stimulate transcription of ERE-driven reporter genes (not shown).

Because alternatively spliced forms of the ERα are present in MCF-7 cells along with the intact receptor, it was of interest to determine whether ERΔE7 interferes with the activity of both wtERα and ERβ. To address this question, we performed titration experiments in which we varied the ratio of wtER to ERΔE7 (Fig. 6). Thus, we transiently transfected HeLa cells with ERα or ERβ expression vectors and increas-

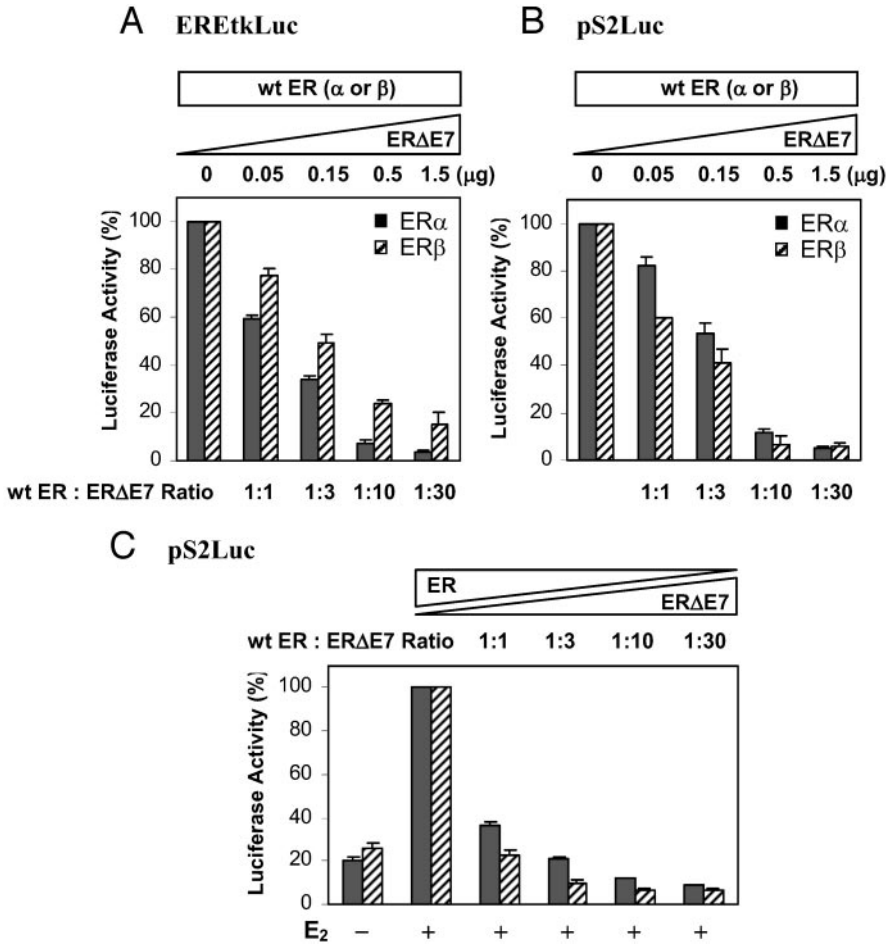

FIG. 6. ERΔE7 inhibits estrogen-dependent transactivation of ERE-driven reporters by ERα and ERβ in a dose-related fashion. HeLa cells were cotransfected with 50 ng expression vectors encoding hERα or mERβ; 0.5 μg ERE-driven reporter plasmids pEREtkLuc (A) or pS2Luc (B); 50 ng of internal control plasmid pRL-TK; and increasing amounts of ERΔE7 expression vector (0–1.5 μg as indicated). The total amount of DNA was held constant to 2.1 μg by addition of empty expression vector, pcDNA3. C, This experiment was carried out with a constant amount of ER expression vectors (0.1 μg total DNA per well) and varying the ratios of wtERs to ERΔE7 as indicated. After transfection, cells were treated with 100 nM E_2 for 24 h and then harvested. Luciferase activities were normalized to the *Renilla* luciferase activities. The data are expressed as the percentage of wtER (α or β) luciferase activity remaining; 100% was assigned to the response obtained with wtER (α or β) plus estradiol. The activation varied from 3- to 5-fold in different experiments. The *bars* represent the mean ± SD of three independent experiments.

ing amounts of ERΔE7 plasmid along with the ERE-driven reporter plasmids pEREtkLuc (Fig. 6A) or pS2Luc (Fig. 6B). A saturating concentration of hormone (100 nM E_2) was used. In both experiments we observed that increasing amounts of ERΔE7 resulted in a progressive inhibition of the E_2-dependent induction of luciferase activity by both ERα and ERβ.

To rule out the possibility that the potent transcriptional suppression observed with high amounts of ERΔE7 was due to high levels of this variant that might poison the transcription apparatus, we performed HeLa transfections using a constant amount of ER expression vectors (0.1 μg per well) and varying the relative amounts of wtERs and ERΔE7 (Fig. 6C). In this case, ERΔE7 also inhibited ERE-driven transcription reaching values to below basal activity. Thus, ERΔE7 appears as a genuine dominant negative inhibitor that did not act by simply saturating the transcription machinery.

ERΔE7 blocks the binding of wtERα and ERβ to their responsive element

To investigate whether inhibition of transcription by ERΔE7 is exerted at level of DNA binding, we compared the ability of ERΔE7 and wild-type receptors to bind to the ERE *in vitro*. For this purpose we conducted EMSAs using whole extracts from HeLa cells that were transfected with wtERα or ERΔE7 independently or with different ratios of both receptors (Fig. 7A). The wtERα formed a complex with the ERE probe that increased in the presence of E_2 (lane 2). The specificity of the retarded band was demonstrated by supershift induction with anti-ER antibodies (lane 3). In contrast, ERΔE7 showed no binding to the ERE in this *in vitro* EMSA (lanes 8 and 9). Interestingly, when both receptors were coexpressed the ERα-ERE complex was attenuated, and this reduction was proportional to the increase of ERΔE7 (lanes 4–7). Similar experiments were performed with extracts from cells expressing ERβ or different ERβ:ERΔE7 ratios (Fig. 7B), and identical results were obtained, suggesting that the transcriptional inhibition by ERΔE7 is arisen from the inhibitory effect of this receptor on the binding of both wtERα and ERβ to ERE.

We also performed a series of EMSA in which wtERα and ERΔE7 were mixed after *in vitro* translation (Fig. 7C). The translational efficiencies of the two synthetic mRNAs were determined to be equivalent by performing parallel reactions in the presence of [^{35}S]-methionine (data not shown). As expected, the wtERα was able to form specific ER-ERE complexes (lane 1), which could be competed out by addition of an excess of unlabeled ERE (lane 2). When ERΔE7 was mixed with wtERα, as the amount of ERΔE7 increased, the binding of wtERα to its response element was progressively inhibited

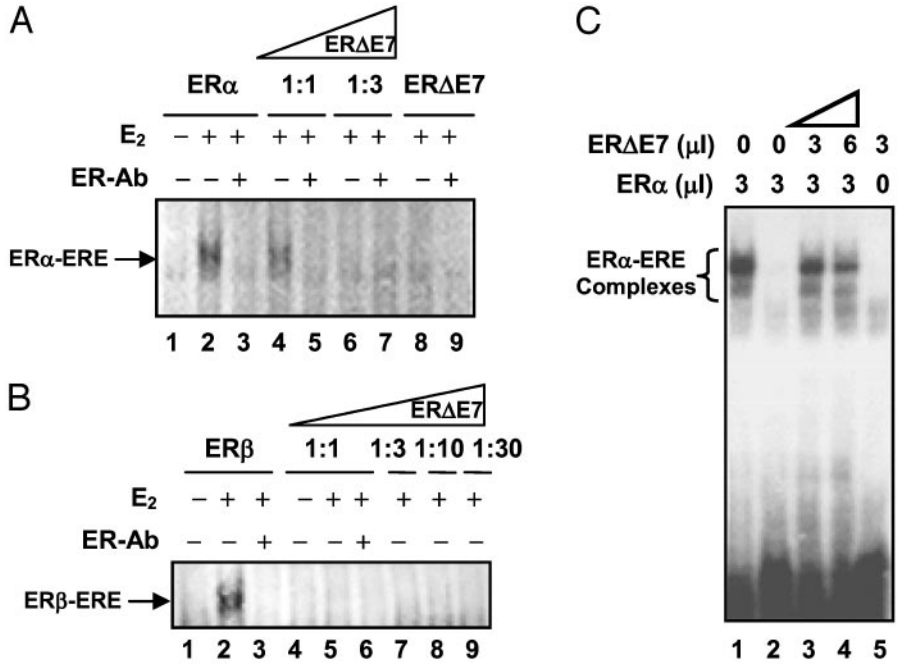

FIG. 7. ERΔE7 inhibits estrogen-dependent binding of both wtERα and ERβ to ERE. A, Whole extracts prepared from transfected HeLa cells expressing ERα, ERΔE7 or 1:1 and 1:3 proportions of both receptors (as indicated) were assayed for ERE-binding activity by EMSA in the absence (−) or presence of 100 nM E_2 (+). Specific ERα-ERE complexes were determined by supershift induction with polyclonal anti-ER antibodies (lanes 3, 5, 7, and 9). B, Whole extracts prepared from transfected HeLa cells expressing ERβ or the indicated proportions of ERβ/ERΔE7 were assayed as above. Specific ERβ-ERE complexes were determined by supershift induction with polyclonal anti-ER antibodies (lanes 3 and 6). C, Three microliters of *in vitro* synthesized wtERα were mixed with increasing amounts of ERΔE7 as indicated. In lane 2, an approximately 100-fold excess of unlabeled ERE was added.

(lanes 3 and 4), even though ERΔE7 was by itself unable to bind to the ERE (lane 5).

AF1-dependent transcriptional activation by wtERα and ERΔE7

The ERΔE7 variant is unable to bind ligand and devoid of ligand-dependent activity and modulation by estrogens and antiestrogens. In spite of all these properties, this variant might activate transcription through steroid-independent mechanisms that involve the AF1 domain, which remains intact. It has been described that the activity of the N-terminal AF1 of ERα is modulated by the phosphorylation of Ser (118) through the Ras-MAPK pathway (11). Thus, epithelial growth factor (EGF) and phorbol-12-myristate-13-acetate (PMA) have been shown to activate ERα (11, 12). On the other hand, it has been shown that estradiol contributes with these agents to the phosphorylation of Ser (118) by a MAPK-independent mechanism (34).

To investigate the E_2-independent activation, we examined the ability of EGF and PMA to activate the transcription mediated by ERΔE7. For this purpose, we conducted transfection experiments in HeLa cells with either wtERα or ERΔE7 to evaluate the transcriptional activity of these receptors on luciferase reporter plasmids containing ERE sites into a synthetic promoter (Fig. 8A) or the natural promoter (Fig. 8B). In the presence of wtERα, EGF and PMA activated transcription 8- to 10-fold, and this effect was potentiated in the presence of E_2. We performed two types of control experiments that clearly demonstrate that the activation with EGF and PMA was mediated by ERα: 1) in the absence of the ER, the expression of luciferase was significantly reduced; and 2) the response was blocked by the antiestrogen ICI. ERΔE7 was not activated by EGF or PMA. This variant showed only a partial activation by PMA on pS2 promoter, which was neither stimulated with E_2 nor abolished by ICI treatment. Although the relative activation of ERΔE7 by PMA appears to be similar to that of wtERα, the level of transcription obtained with ERΔE7 represented about 50% of that of wtERα. These findings suggest that the integrity of the AF1 region of ERΔE7 is not sufficient to support full activation by EGF and PMA. This is in fact not too surprising because ERΔE7 fails to bind ERE.

Discussion

The presence of ER variants has been shown in human breast cancer tissues (7), breast cancer cell lines (8), and a number of human normal and neoplasmic tissues (7, 9, 35, 36). Recently, using a splice-targeted primer approach, 20 alternatively spliced ERα mRNAs that are present in both breast cancer cell lines and tumors have been identified (8). Information on these variants, however, is limited to *in vitro* analysis at the mRNA levels. The biological significance of the alternatively spliced messengers remains an enigma. It is possible that variant receptor proteins are defective in folding, dimerization, or interaction with heat shock proteins (37) or other cellular factors (38) that may lead to rapid degradation. Thus, Dauvois *et al.* (39) have reported that impaired dimerization leads to a decreased half-life of the receptor protein. Characterization of variant receptor proteins is just beginning to emerge. Translation into protein has been shown for only ERΔE4 (40) and ERΔE5 (19, 41). Recently, a 52-kDa protein corresponding to ERΔE7 was detected by Western analysis and shown to be the major variant ER protein expressed in various ER (+) breast cancer cell lines and extracts from ER (+) breast and uterine tumors (42). In addition, ERΔE7 mRNA has been reported to be the major alternatively spliced form in most human breast tumors and cancer cell lines as well as in human uterus and endometrial tumors (43, 44).

Up to now, no function has been established for the ERΔE7 variant in mammalian cells. Two reports indicated that

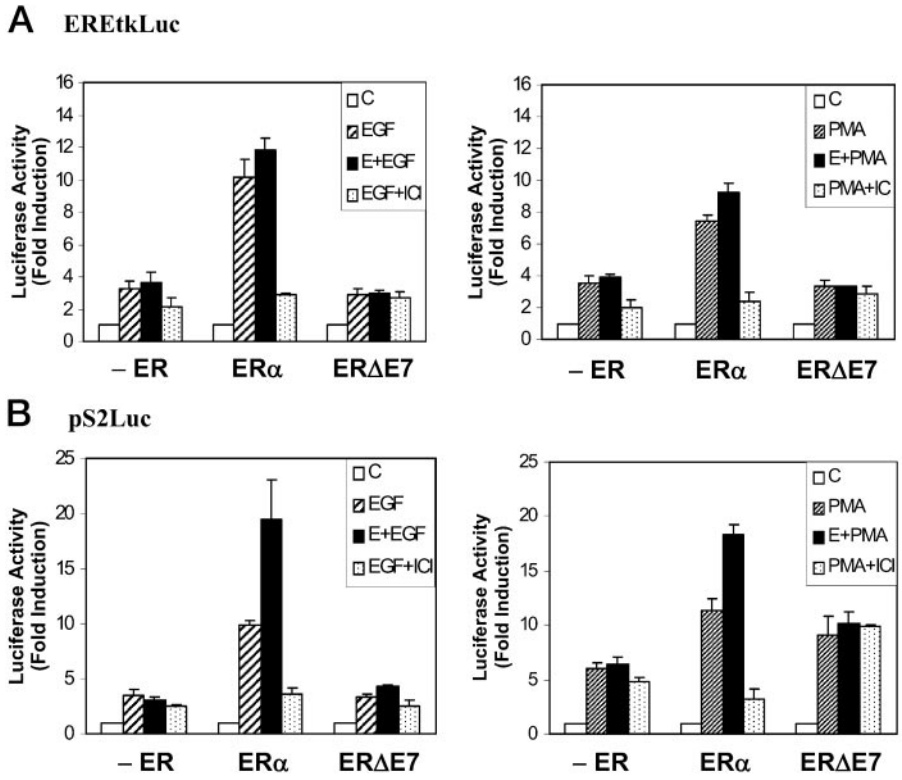

FIG. 8. Ligand-independent transcriptional activation of wtERα and ERΔE7. HeLa cells were cotransfected with 100 ng expression vectors encoding ERα or ERΔE7 or empty expression vector, pcDNA3 (indicated as −ER); 0.5 μg ERE-driven reporter plasmids, pEREtkLuc (*A*) or pS2Luc (B); and 50 ng of internal control plasmid pRL-TK. When indicated, the transfected cells were treated with 100 ng/ml EGF, 100 nM PMA, 100 nM E_2, 1 μM ICI, or combinations of them. Luciferase activities were normalized to the *Renilla* luciferase activities. The data are reported as fold induction with respect to untreated cells (C), to which it was assigned the value 1. The *bars* represent the mean ± SD of three separate experiments.

ERΔE7 is a dominant inhibitor of wtERα function in yeast (23, 24). In this work we have approached the functional characterization of this variant receptor obtained from MCF-7 cells. ERΔE7 represents the prevalent spliced form of ERα expressed in this breast carcinoma-derived cell line, as determined at both mRNA and protein levels. Our studies provide evidence for the first time that ERΔE7 suppresses the estrogen-dependent transcriptional activation by both wtERα and ERβ. At a 1:1 ratio, the suppression of estrogen-induced transcription varied from 20–40%, depending on the wtER and ERE promoter used. Increasing amounts of ERΔE7 resulted in the progressive inhibition of E_2-dependent response, achieving a complete inhibition at a 10-fold molar excess of ERΔE7. This observation may have physiological significance in breast cancer cells that predominantly express this ERα variant.

Powerful dominant negative mutants generated by chemical mutagenesis of the ERα LBD have been described (45). ERΔE7 has the additional interest that it is a naturally occurring ER variant that may have profound effects on several estrogen-dependent functions if expressed at high levels relative to wtERs.

With regard to the selective modulation observed using two different ERE promoters, it has been reported that the EREs may act as allosteric modulators of ER conformation. Thus, the *Xenopus* vitellogenin A2 ERE, (GGTCAnnnTGACC) and the human pS2 ERE (GGTCAnnnTGGCC) induce changes in receptor conformation that could lead to association of the receptor with different transcription factors and assist in the differential modulation of estrogen-responsive genes in target cells (46).

The dominant negative character of ERΔE7 suggests that this variant is able to interact with at least one component of the ERE-directed transcription complex in a manner that disrupts positive gene regulation mediated by both ERα and ERβ. Based on gel mobility shift assays, ERΔE7 is unable to bind to ERE by itself, and it prevents both wtERα and ERβ from binding to DNA. This refutes the results of Fuqua *et al.* (23), who claimed to identify ERΔE7 by complex formation with ERE and upshift induction with anti-ER antibody H222. It is unlikely that the protein detected was ERΔE7, which is now known not to react with H222 (42). We cannot rule out a weak protein-DNA interaction, which would not be detected in a gel shift assay. In any case, it is clear that the ability of ERΔE7 to suppress the activity of wtER is not due to the high affinity of the former for the ERE. Additionally, ERΔE7 might form inactive heterodimers with wtERs. These heterodimers could be unable to either bind to the ERE or activate transcription when bound to the ERE. We found that ERΔE7 can form a stable complex with ERα in a ligand-independent manner, as expected by the inability of ERΔE7 to bind ligands. This is consistent with the absence of allosteric modulation of ERΔE7 by estrogens and antiestrogens but disagrees with other observations using the two-hybrid system in yeast in which ERΔE7 could form neither homodimers nor heterodimers with wtERα (24). Identical conclusions can be drawn from immunoprecipitation studies using *in vitro* translated ERα and ERβ. Although immunoprecipitation assays are extremely difficult to use to quantitate the percentages of the various heterodimers that are immunoprecipitated, our data clearly demonstrate that heterodimers with and without ligand are immunoprecipitated to a similar extent. Thus, ligand binding is not a prerequisite for receptor dimerization, as indicated also by Zhuang *et*

al. (47). Finally, Far-Western analysis also showed that wtERα can form heterodimers with ERΔE7 with the same efficiency that it can form homodimers. Altogether, these studies show that ERΔE7 is able to form mixed dimers with both wtERα and ERβ and these heterodimers are unable to bind stably to DNA.

The ERΔE7 variant might also interfere with associated transcription factors required for ER activity. Using pull-down experiments, we determined that the estrogen-dependent association of both SRC-1a and AIB1 coactivators with the AF2 domain of wtERα is prevented in the truncated AF2 of the ERΔE7 variant. This result is in agreement with those reported by Heery *et al.* (48), who showed that the ability of SRC-1 to bind the ER and enhance its transcriptional activity is dependent on the integrity of the LXXLL motifs and on key hydrophobic residues in the conserved helix 12 of the ER. ERΔE7 lacks this essential region.

Like other nuclear receptors, ERα is a modular protein in which individual domains are capable of demonstrating autonomous functions (10, 32). It can reasonably be assumed that the exclusion of a particular exon will result in a protein lacking the function ascribed to that exon. Alternatively, it is possible that the loss of a particular exon will result in unpredictable functional deficits or perhaps even bestow a novel function on the variant receptor. Some properties determined for ERΔE7 are consistent with these predictions. Thus, the inability of ERΔE7 to bind ligands, its insensitivity to estrogens and antiestrogens, and the lack of association with coactivators is not surprising because the loss of exon 7 implies the elimination of a significant portion of HBD/AF2 domain including helix 12. Less predictably, although ERΔE7 contains both the DNA-binding domain and AF1 domains, this receptor shows a strong defect in ERE recognition and DNA binding and therefore the loss of AF1-dependent activation by EGF and PMA. Our results indicate that AF1 and AF2 exert mutual influence because the loss of AF2 in ERΔE7 affects transactivation through AF1. It has been indicated that mutations in or near the AF2 transactivation region or elimination of the AF2 region are responsible for the dominant negative phenotype of the C-terminal ER mutants, whereas ER mutants made inactive by mutations in the hormone-binding region did not possess the capacity to act as effective blockers of ER action (45). These observations reinforce the idea that it is the disruption of the transactivation domain, and not the loss of ligand binding, that leads to the dominant negative phenotype exhibited by ERΔE7.

Variant forms of the ER that function as dominant negative may play an important role in the loss of hormone responsiveness and the progression to hormone independence. The existence of different variants generated by alternative splicing of ERα and ERβ that function as dominant negative has been interpreted as a physiological protective mechanism of regulating the E_2-dependent growth of responsive tissues (49) and, alternatively, as a deleterious mechanism that render the ER+ cancer cells resistant to antiestrogen therapy (23, 50).

Because the growth of nearly 50% of all human breast cancers is dependent on the presence of an active estradiol-ER complex, it is interesting to explore ways to functionally inactivate ERs (51). In this regard, elevated levels of a dominant negative receptor that interferes with normal ER function could render a tumor unresponsive to estrogen and antiestrogens (23). Thus, the ERΔE7 variant is significantly more abundant in ER+/PgR− tumors, compared with ER+/PgR+ tumors (23), being the ER+/PgR− phenotype more aggressive tumors, growing much faster and with a lower response to antihormone therapy (52). Also, it has been shown that the estrogen-independent LCC2 cells express significantly higher levels of ERΔE7 transcripts, compared with the estrogen-dependent MCF-7 cells (53).

The studies of ER variants that have been published thus far been aimed at the identification of possible causes of the hormone-independent and antihormone-resistant growth of human breast cancers. Perhaps too little attention has been paid to establish the relative wtER/variant ratio of expression in both normal and tumoral tissues, a circumstance that in our view severely conditions the possible involvement of ER variants in physiological and/or pathological processes.

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