

# Cross-inhibition of both estrogen receptor $\alpha$ and $\beta$ pathways by each dominant negative mutant

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Received 16 January 1998

**Abstract** Both estrogen receptor  $\alpha$  (ER $\alpha$ ) and the recently identified ER $\beta$  are nuclear receptors that are activated by estrogen. It was reported that ER $\alpha$  and ER $\beta$  form heterodimers. Here, we show that they activate transcription independently rather than synergistically via estrogen response elements (ERE). To show the cross-talk between ER $\alpha$  and ER $\beta$ , we utilized dominant negative mutants of ERs constructed by C-terminal truncation. Interestingly, ER $\alpha$ 1–530 inhibited transactivation not only by ER $\alpha$  but also by ER $\beta$ , whereas ER $\beta$ 1–481 inhibited transactivation by ER $\alpha$  as well as by ER $\beta$ . The GST pull-down assay also demonstrated the cross-interaction of these mutants with wild-type ER $\alpha$  and ER $\beta$ . Thus, we found dominant negative mutants that block both ER $\alpha$  and ER $\beta$  signaling pathways.

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**Key words:** Estrogen; Estrogen receptor; Heterodimer; Transactivation; Human

## 1. Introduction

Estrogen is known to exert striking effects on the growth and development of the reproductive organs. It is assumed that its diverse effects are mediated in major part by the estrogen receptor (ER), a member of the nuclear receptor superfamily [1,2].

Recently, a second ER, namely ER $\beta$ , has been isolated in rat prostate [3], human testis [4] and mouse ovary [5]. ER $\beta$  displays high affinity binding of 17 $\beta$ -estradiol (E<sub>2</sub>). The DNA binding domain of ER $\beta$  is almost identical to ER $\alpha$ , implying that both ER $\alpha$  and ER $\beta$  share the same DNA response element. More recently, the heterodimer complex of ER $\alpha$  and ER $\beta$  has been shown in vitro and in vivo irrespective of ligand binding. Furthermore, ER $\alpha$ /ER $\beta$  heterodimers bind to an ERE with an affinity similar to that of the ER $\alpha$  homodimer and greater than that of the ER $\beta$  homodimer [6,7]. However, there is no functional evidence of ER $\alpha$ /ER $\beta$  heterodimers in vivo. On the other hand, in the case of an ER $\alpha$  C-terminal truncation mutant, ER $\alpha$ 1–530 was reported to have a dominant negative activity against wild-type ER $\alpha$  [8]. The constitutive negative phenotype of ER $\alpha$ 1–530 is at least partly because its activation function 2 (AF-2) is eliminated.

In order to investigate the cross-talking possibility of ER $\alpha$  and ER $\beta$  by their functional heterodimerization, we constructed ER $\alpha$ 1–530 and its equivalent mutant of ER $\beta$  (ER $\beta$ 1–481) to study the inhibition of each mutant against wild-type ER $\alpha$  and ER $\beta$ . As a result, ligand-dependent trans-

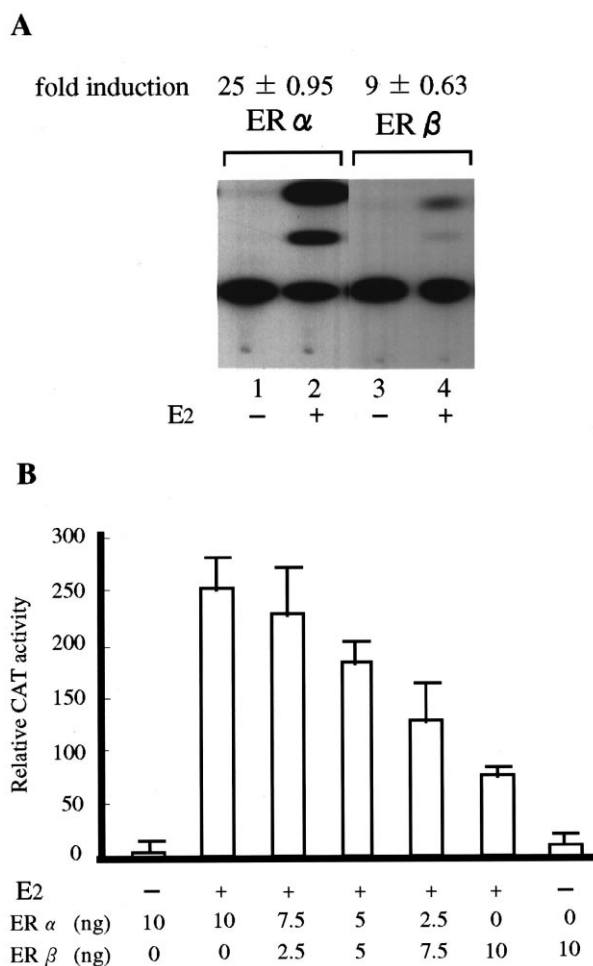


Fig. 1. E<sub>2</sub>-dependent transactivation of co-expressed ER $\alpha$  and ER $\beta$ . A: Transactivation of the ERE-GCAT reporter by ER $\alpha$  and ER $\beta$ . COS-7 cells were transfected with 2  $\mu$ g of ERE-GCAT reporter plasmid and 10 ng of wild-type ER expression vectors. The transfected cells were incubated for 24 h in the absence (–) or presence (+) of 10<sup>–7</sup> M E<sub>2</sub>. Then CAT activities were determined and normalized relative to the  $\beta$ -galactosidase activities expressed by the PCH110 internal control vector. CAT activities are indicated as the fold induction of the control, and are presented as means±standard deviations calculated from three independent experiments. B: pCXN2-hER $\alpha$  and pCXN2-hER $\beta$  were co-transfected with EREG-CAT in COS-7 cells in varying ratios from 0 to 10 ng.

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**Abbreviations:** ER, estrogen receptor; ERE, estrogen response element; RT-PCR, reverse transcription polymerase chain reaction; UTR, untranslated region; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; AF-2, activation function 2

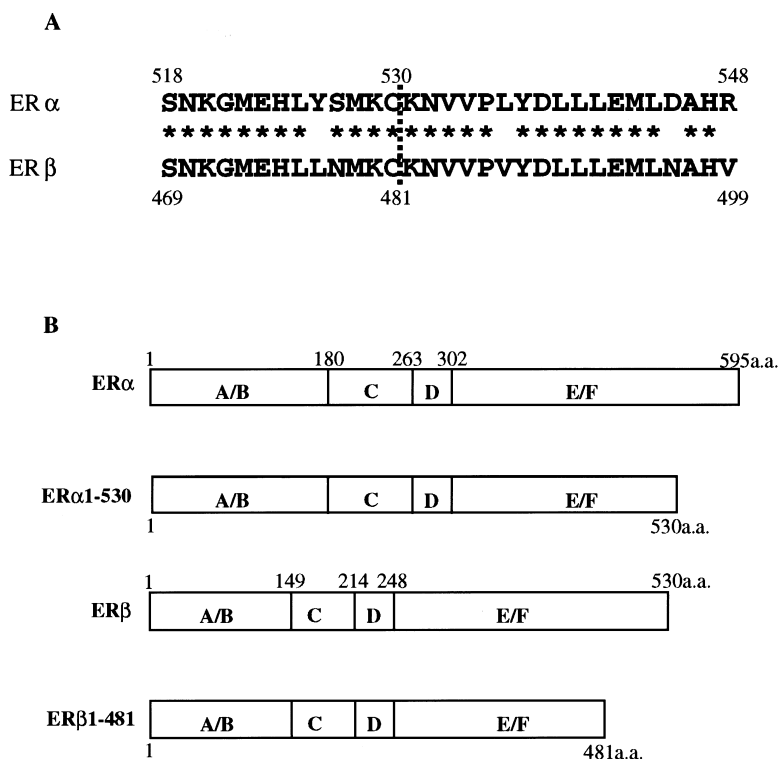


Fig. 2. Structures of ER $\alpha$ 1–530 and ER $\beta$ 1–481. A: Comparison around the C-terminal truncated portion of ER $\alpha$ 1–530 and ER $\beta$ 1–481. The human ER $\alpha$  deduced amino acid sequence (shown above) and corresponding ER $\beta$  amino acids (shown below) are depicted. Asterisks indicate identity between the sequences. B: Comparison between ER $\alpha$ 1–530, ER $\beta$ 1–481 and wild-type ER $\alpha$  and ER $\beta$  proteins. The functional A–E/F domains are schematically represented, with the numbers of amino acid residues as indicated.

activation of ER $\alpha$  and ER $\beta$  on an ERE was suppressed by each of their C-terminal truncated mutants, suggesting the cross-talking function of ER $\alpha$ /ER $\beta$  heterodimers in vivo.

## 2. Materials and methods

### 2.1. Plasmid construction

The ER $\alpha$  cDNA originating from HEG0 [9] was cloned into the pCXN2 expression vector [10] to construct pCXN2-hER $\alpha$ . The ER $\alpha$  cDNA encoding amino acids 117–595 was cloned into pGEX4T-2 (Pharmacia) to construct GST-hER $\alpha$ . The RT-PCR product of ER $\beta$  cDNA (nucleotides 1–1740) [11] was inserted into pCXN2 and into pGEX4T-2 (Pharmacia) to construct pCXN2-hER $\beta$  and GST-hER $\beta$ , respectively. The PCR-amplified products of ER $\beta$ 1–481 (nucleotides 99–1541) and ER $\alpha$ 1–530 (cDNA fragment encoding amino acids 1–530) were cloned into pCXN2 to construct pCXN2-hER $\beta$ 1–481 and pCXN2-hER $\alpha$ 1–530, respectively. Construction of ERE-GCAT was described elsewhere [12]. All constructs were verified by sequencing.

### 2.2. DNA sequence and analysis

The nucleotide sequences were determined by sequencing of both strands of alkaline-denatured plasmid DNA using the BcaBest sequencing kit (TaKaRa Co.). The obtained DNA sequence was compiled and analyzed using DNASIS computer programs (Hitachi Co.).

### 2.3. Cell transfection and preparation of whole cell extracts

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 10% dextran-coated charcoal-treated fetal calf serum [13].  $5 \times 10^5$  cells in 10 cm Petri dishes were transfected with a total of 20  $\mu$ g of plasmids using calcium phosphate [14]. Cells were harvested 36 h after transfection and whole cell extracts were prepared by freeze-thawing and diluted in 100  $\mu$ l of TEG buffer (10 mM Tris, pH 7.5, 1.5 mM EDTA, 10% glycerol).

### 2.4. Chloramphenicol acetyltransferase (CAT) assays

The CAT assay was performed as described [15]. Briefly,  $5 \times 10^5$  of COS-7 cells were transfected with a total of 15  $\mu$ g of DNA. 2  $\mu$ g of ERE-GCAT reporter plasmid was co-transfected with indicated amounts of receptor expression vectors. All assays were performed in the presence of 2  $\mu$ g of PCH110 (Pharmacia), a  $\beta$ -galactosidase expression vector used as internal control to normalize for variations in transfection efficiency. The total amount of DNA and expression vectors for transfection was adjusted using pGEM3Zf (Promega) and pCXN2, respectively. After 12 h incubation with the calcium phosphate-precipitated DNA, the cells were washed with fresh medium and incubated for an additional 24 h in the absence or presence of  $10^{-7}$  M E<sub>2</sub>. Cell extracts were prepared by freeze-thawing and assayed for CAT after normalization for  $\beta$ -galactosidase activity.

### 2.5. Glutathione S-transferase (GST) pull-down assay

ER $\alpha$  and ER $\beta$  proteins were synthesized in vitro using the TnT-coupled reticulocyte lysate system (Promega). GST, GST-hER $\alpha$  and GST-hER $\beta$  proteins were induced, solubilized, and bound to glutathione beads following the manufacturer's instruction (Pharmacia LKB). After binding to glutathione beads, 15  $\mu$ l of the suspension was incubated with 1–2  $\mu$ l of the appropriate <sup>35</sup>S-labeled, in vitro translated protein for 1 h in 500  $\mu$ l of NETN (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). Following incubation, the beads were washed three times with NETN. Bound proteins were eluted with 20  $\mu$ l of 1 $\times$ SDS-PAGE buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and electrophoretically separated in a SDS/7.5% polyacrylamide gel.

## 3. Results

In order to investigate the possibility that ER $\alpha$  and ER $\beta$  mutually enhance the E<sub>2</sub>-dependent transcriptional activity on an ERE, pCXN2-hER $\alpha$  and pCXN2-hER $\beta$  were co-trans-

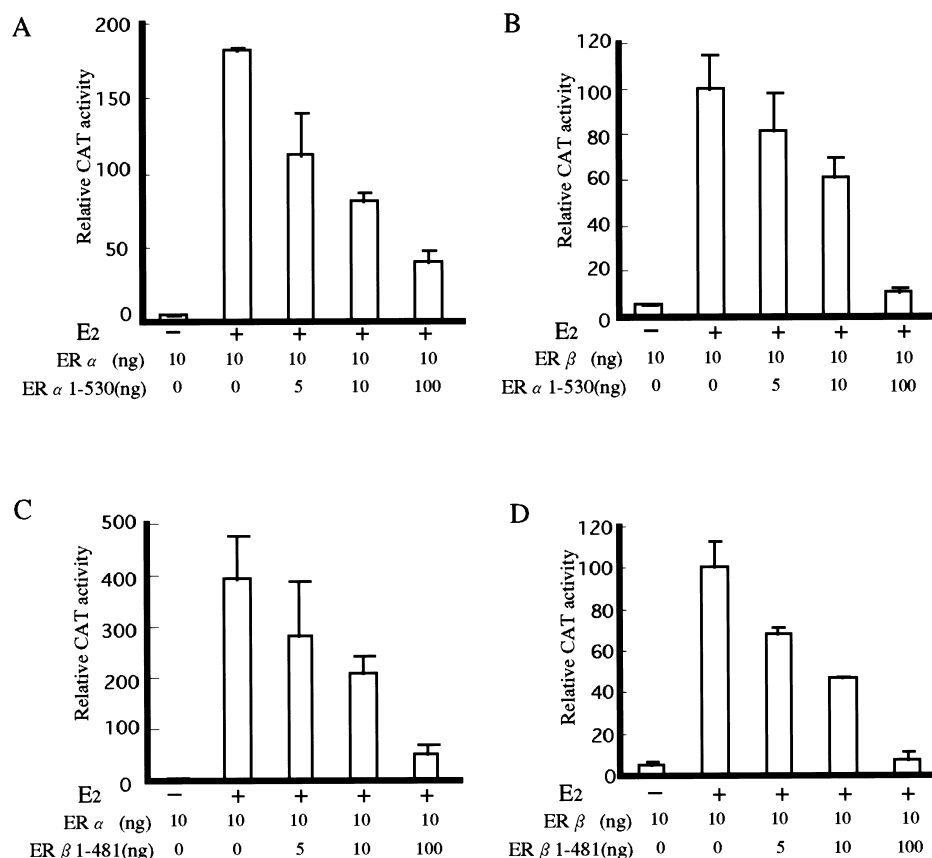


Fig. 3. Inhibitory effects of ERα1-530 and ERβ1-481 against the transactivation by wild-type ERs. Dose-dependent inhibitory effects of ERα1-530 against the transactivations by ERα (A) and ERβ (B) of the ERE-GCAT reporter are shown by CAT assay. Dose-dependent inhibitory effects of ERβ1-481 against the transactivations by ERα (C) and ERβ (D) of the ERE-GCAT reporter are also shown. ERα, ERβ, ERα1-530 and ERβ1-481 are the abbreviations of pCXN2-hERα, pCXN2-hERβ, pCXN2-hERα1-530 and pCXN2-hERβ1-481, respectively.

ected with EREG-CAT reporter construct (see Section 2). In COS-7 cells transfected with only ERα or ERβ, relative CAT activity increased 25-fold and 9-fold, respectively, upon stim-

ulation with  $10^{-7}$  M E<sub>2</sub> (Fig. 1A). Transcriptional activity of various ratios of co-expressed ERα and ERβ was shown in Fig. 1B.

Previous studies have shown that ERα1-530 acts as a dominant negative mutant against ERα transactivation [8]. Here, we constructed ERα1-530 expression vector and its equivalent ERβ mutant from the sequence homology around their truncation sites (Fig. 2A), that is ERβ1-481 as shown in Fig. 2B. Interestingly, ERα1-530 inhibited the ligand-dependent transactivation by ERβ (Fig. 3B) as well as that by ERα (Fig. 3A). Moreover, ERβ1-481 inhibited not only the transactivation by ERβ (Fig. 3D) but also that by ERα (Fig. 3C).

In vitro translated products of ERα1-530 consisted of two proteins of 59 kDa and 48 kDa, both of which were pulled down using the GST-fused ERα or ERβ protein (Fig. 4, lanes 1, 5, 7). As for ERβ1-481 products, 54 kDa and 51 kDa proteins were detected, both of which were pulled down by GST-hERα or hERβ protein (Fig. 4, lanes 2, 6, 8). In vitro translated ERα1-530 and ERβ1-481 were not pulled down with the GST protein alone (Fig. 4, lanes 3, 4).

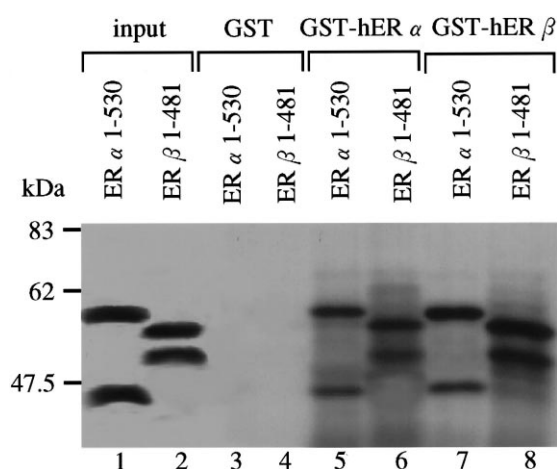


Fig. 4. GST pull-down assay. ERα1-530 and ERβ1-481 proteins were synthesized in vitro using the TnT-coupled reticulocyte lysate system (Promega), shown in lanes 1 and 2 at the amount of 10% of input. After binding of GST, GST-hERα and GST-hERβ proteins to glutathione beads, 15 μl of the suspension was incubated with 1–2 μl of the appropriate <sup>35</sup>S-labeled, in vitro translated protein for 1 h in 500 μl of NETN buffer. Bound proteins were eluted and electrophoretically separated in a SDS/7.5% polyacrylamide gel.

#### 4. Discussion

Recently, we [11] and others [6,7] demonstrated the heterodimerization between ERα and ERβ in vivo and in vitro. Since the formation of ERα/ERβ heterodimers was established, we then tried to ascertain the functional interaction

between ER $\alpha$  and ER $\beta$  in vivo. Co-transfection experiments have shown that they enhance the mutual transcriptional activity independently rather than synergistically.

In order to demonstrate their functional heterodimerization, we utilized the dominant negative activities of the C-terminal truncated ER mutants, ER $\alpha$ 1–530 and ER $\beta$ 1–481, against wild-type ER $\alpha$  and ER $\beta$ . Both mutants were found to inhibit the transactivation by ER $\alpha$  as well as by ER $\beta$ . The inhibitory effect of C-terminal truncated ER mutants may be explained by the following three mechanisms: competition for ERE binding, formation of inactive heterodimers with wild-type ER and specific transcriptional silencing [8,16]. All these mechanisms are possible for their dominant negative effect and they are not mutually exclusive. Because ER $\alpha$ 1–530 retains the region important for ER dimerization (500–520 aa) [17] and ER $\beta$ 1–481 also possesses the corresponding region, their interactions with wild-type ERs would be intact. To show heterodimerization between these mutant ERs and wild-type ERs, the GST pull-down assay was performed. Because GST-fused full-length ER $\alpha$  was not soluble, we here utilized GST fusion protein with N-terminal truncated ER $\alpha$ . The protein interactions were demonstrated clearly as shown in Fig. 4. Thus, it was shown that both ER $\alpha$ 1–530 and ER $\beta$ 1–481 can act by heterodimerization with wild-type ER $\alpha$  as well as ER $\beta$ , at least. The sizes of the ER $\beta$ 1–481 protein translated in vitro were approximately 54 kDa and 51 kDa, suggesting its translational initiation from both the first ATG and its downstream ATGs. As for ER $\alpha$ 1–530, the 58 kDa protein was translated in vitro as well as the 47 kDa protein, both of which were detectable by the anti-ER $\alpha$  monoclonal antibody D547 [18,19] (data not shown). The 47 kDa protein might be translated from downstream ATGs of ER $\alpha$ 1–530 or its degradation product.

To characterize the cross-talk between ER $\alpha$  and ER $\beta$  in detail, it is also necessary to investigate the distinct functions of ER $\alpha$  and ER $\beta$ . The homology of the N-terminal A/B domains including the activation function 1 (AF-1) of ER $\alpha$  and ER $\beta$  is only 30% [11], which might be responsible for the selectivity of the tamoxifen antagonism only observed in ER $\alpha$  [12]. Moreover, E<sub>2</sub>-dependent transactivation properties of ER $\alpha$  and ER $\beta$  on AP1 were recently reported to be in opposite modes [20]. The specificity of the transactivational regulation between ER $\alpha$  and ER $\beta$  was also reported in terms of the distinct activity of cofactors, i.e. SRC-1 [5]. These findings suggest the possibility that different transactivational mechanisms are present in ER $\alpha$  and ER $\beta$  because of their different AF-1 and AF-2 activities. Heterodimerization of these receptors would provide another dimension to the complex regulation of target tissues by estrogen, which is yet to be elucidated.

In conclusion, we have made ER $\alpha$  and ER $\beta$  mutants which block the function of both ER $\alpha$  and ER $\beta$  at the transactivation level. Moreover, we have shown evidence for the first time for the cross-interaction of these mutants with wild-type ER $\alpha$  and ER $\beta$ . These dominant negative mutants described here may be utilized as a tool to investigate the physiological role of estrogen receptor in vivo, inhibiting both signal pathways of ERs.

**Acknowledgements:** We thank Dr. Chambon for the gift of HEGO and Dr. Greene for the gift of D547 monoclonal antibody. S.O. was supported by a JSPS Research Fellowship for Young Scientists.

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