Cross-inhibition of both estrogen receptor α and β pathways by each dominant negative mutant

Sumito Ogawa[a,b], Satoshi Inoue[a,b], Akira Orimo[a], Takayuki Hosoi[b], Yasuyoshi Ouchi[b], Masami Muramatsu[a,*]

[a] *Department of Biochemistry, Saitama Medical School, 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-04, Japan*
[b] *Department of Geriatrics, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received 16 January 1998

Abstract Both estrogen receptor α (ERα) and the recently identified ERβ are nuclear receptors that are activated by estrogen. It was reported that ERα and ERβ form heterodimers. Here, we show that they activate transcription independently rather than synergistically via estrogen response elements (ERE). To show the cross-talk between ERα and ERβ, we utilized dominant negative mutants of ERs constructed by C-terminal truncation. Interestingly, ERα1–530 inhibited transactivation not only by ERα but also by ERβ, whereas ERβ1–481 inhibited transactivation by ERα as well as by ERβ. The GST pull-down assay also demonstrated the cross-interaction of these mutants with wild-type ERα and ERβ. Thus, we found dominant negative mutants that block both ERα and ERβ signaling pathways.

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 ERβ, has been isolated in rat prostate [3], human testis [4] and mouse ovary [5]. ERβ displays high affinity binding of 17β-estradiol (E_2). The DNA binding domain of ERβ is almost identical to ERα, implying that both ERα and ERβ share the same DNA response element. More recently, the heterodimer complex of ERα and ERβ has been shown in vitro and in vivo irrespective of ligand binding. Furthermore, ERα/ERβ heterodimers bind to an ERE with an affinity similar to that of the ERα homodimer and greater than that of the ERβ homodimer [6,7]. However, there is no functional evidence of ERα/ERβ heterodimers in vivo. On the other hand, in the case of an ERα C-terminal truncation mutant, ERα1–530 was reported to have a dominant negative activity against wild-type ERα [8]. The constitutive negative phenotype of ERα1–530 is at least partly because its activation function 2 (AF-2) is eliminated.

In order to investigate the cross-talking possibility of ERα and ERβ by their functional heterodimerization, we constructed ERα1–530 and its equivalent mutant of ERβ (ERβ1–481) to study the inhibition of each mutant against wild-type ERα and ERβ. As a result, ligand-dependent trans-

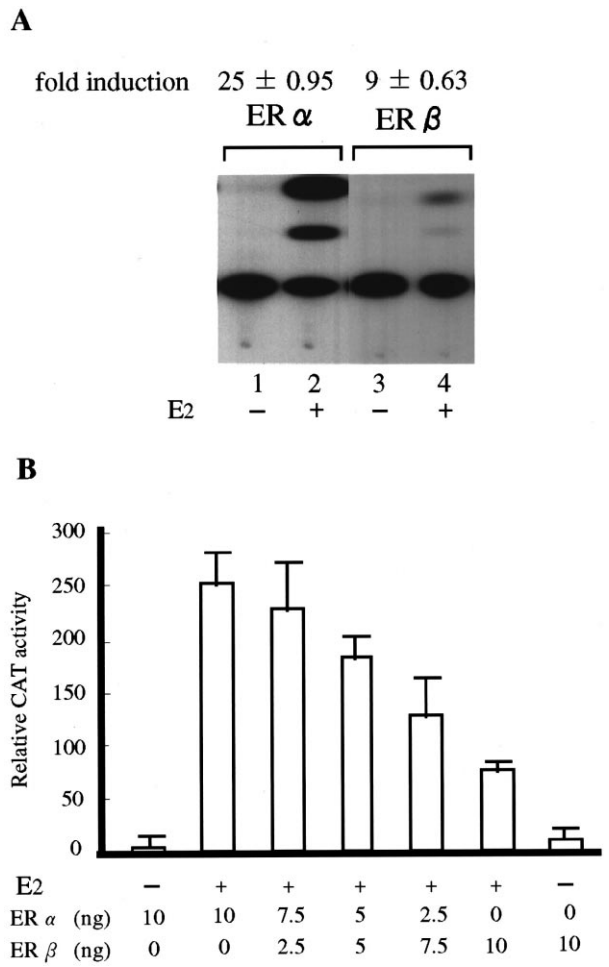

Fig. 1. E_2-dependent transactivation of co-expressed ERα and ERβ. A: Transactivation of the ERE-GCAT reporter by ERα and ERβ. COS-7 cells were transfected with 2 μg of ERE-GCAT reporter plasmid and 10 ng of wild-type ER expression vectors. The transfected cells were incubated for 24 h in the absence (−) or presence (+) of 10^{-7} M E_2. Then CAT activities were determined and normalized relative to the β-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-hERα and pCXN2-hERβ were co-transfected with EREG-CAT in COS-7 cells in varying ratios from 0 to 10 ng.

*Corresponding author. Fax: (81) (492) 94-9751.
E-mail: muramasa@saitama-med.ac.jp

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

PII S0014-5793(98)00079-9

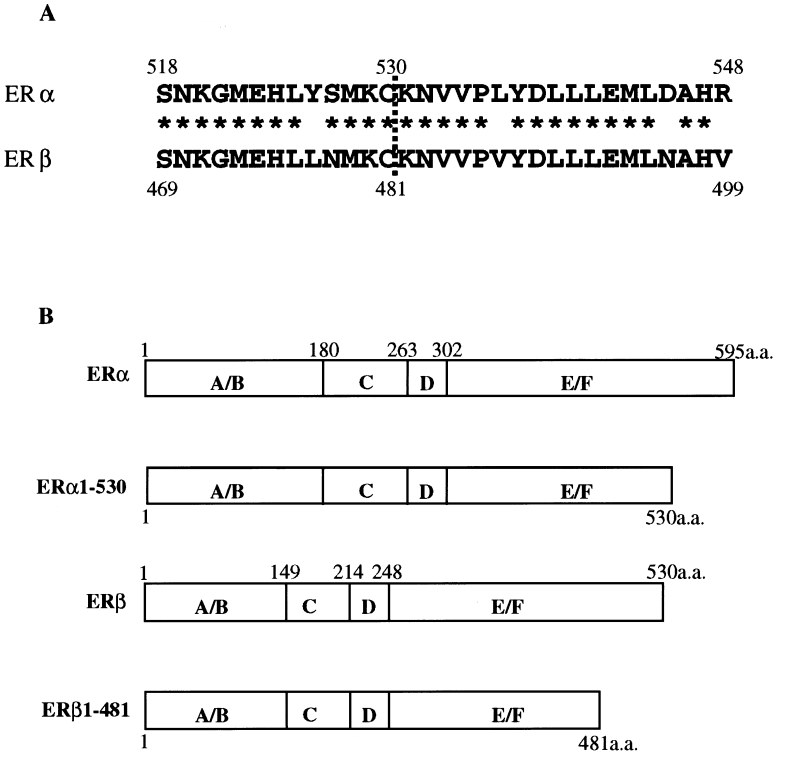

Fig. 2. Structures of ERα1–530 and ERβ1–481. A: Comparison around the C-terminal truncated portion of ERα1–530 and ERβ1–481. The human ERα deduced amino acid sequence (shown above) and corresponding ERβ amino acids (shown below) are depicted. Asterisks indicate identity between the sequences. B: Comparison between ERα1–530, ERβ1–481 and wild-type ERα and ERβ proteins. The functional A–E/F domains are schematically represented, with the numbers of amino acid residues as indicated.

activation of ERα and ERβ on an ERE was suppressed by each of their C-terminal truncated mutants, suggesting the cross-talking function of ERα/ERβ heterodimers in vivo.

2. Materials and methods

2.1. Plasmid construction

The ERα cDNA originating from HEG0 [9] was cloned into the pCXN2 expression vector [10] to construct pCXN2-hERα. The ERα cDNA encoding amino acids 117–595 was cloned into pGEX4T-2 (Pharmacia) to construct GST-hERα. The RT-PCR product of ERβ cDNA (nucleotides 1–1740) [11] was inserted into pCXN2 and into pGEX4T-2 (Pharmacia) to construct pCXN2-hERβ and GST-hERβ, respectively. The PCR-amplified products of ERβ1–481 (nucleotides 99–1541) and ERα1–530 (cDNA fragment encoding amino acids 1–530) were cloned into pCXN2 to construct pCXN2-hERβ1–481 and pCXN2-hERα1–530, 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]. 5×10^5 cells in 10 cm Petri dishes were transfected with a total of 20 μg 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 μl 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, 5×10^5 of COS-7 cells were transfected with a total of 15 μg of DNA. 2 μg of ERE-GCAT reporter plasmid was co-transfected with indicated amounts of receptor expression vectors. All assays were performed in the presence of 2 μg of PCH110 (Pharmacia), a β-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_2. Cell extracts were prepared by freeze-thawing and assayed for CAT after normalization for β-galactosidase activity.

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

ERα and ERβ proteins were synthesized in vitro using the TnT-coupled reticulocyte lysate system (Promega). GST, GST-hERα and GST-hERβ proteins were induced, solubilized, and bound to glutathione beads following the manufacturer's instruction (Pharmacia LKB). After binding to glutathione beads, 15 μl of the suspension was incubated with 1–2 μl of the appropriate ^{35}S-labeled, in vitro translated protein for 1 h in 500 μl 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 μl of 1×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 ERα and ERβ mutually enhance the E_2-dependent transcriptional activity on an ERE, pCXN2-hERα and pCXN2-hERβ were co-trans-

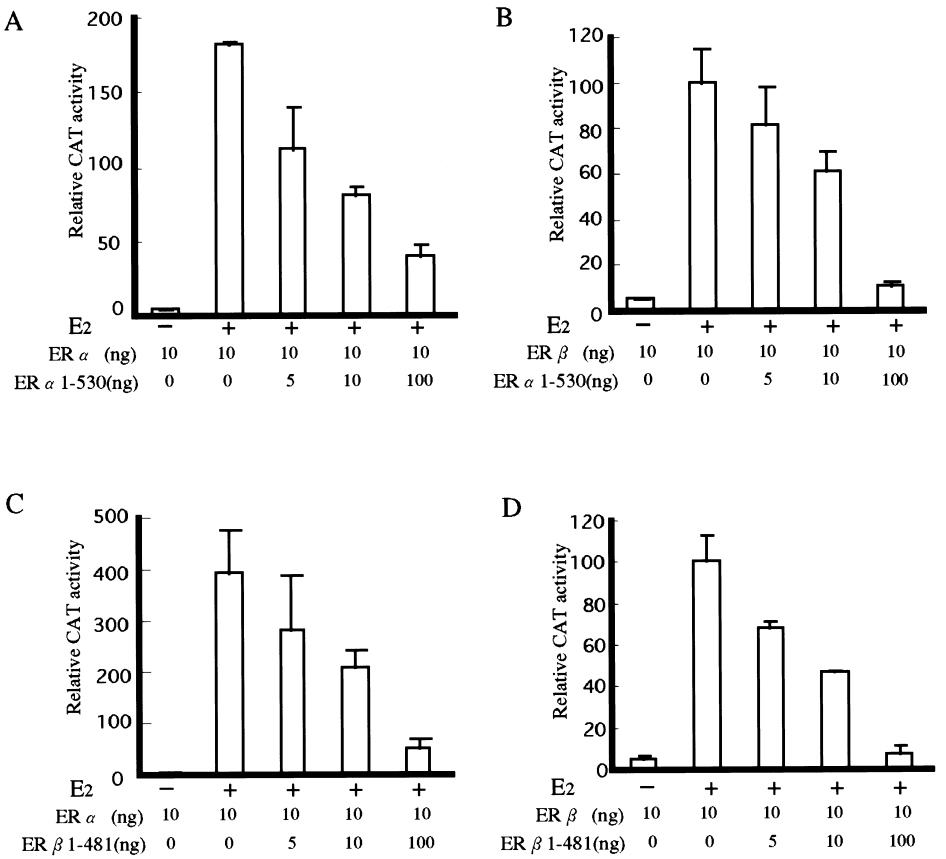

Fig. 3. Inhibitory effects of ERα1–530 and ERβ1–481 against the transactivation by wild-type ERs. Dose-dependent inhibitory effects of ERα1–530 against the transactivations by ERα (A) and ERβ (B) of the ERE-GCAT reporter are shown by CAT assay. Dose-dependent inhibitory effects of ERβ1–481 against the 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.

fected 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 stimulation with 10^{-7} M E_2 (Fig. 1A). Transcriptional activity of various ratios of co-expressed ERα and ERβ was shown in Fig. 1B.

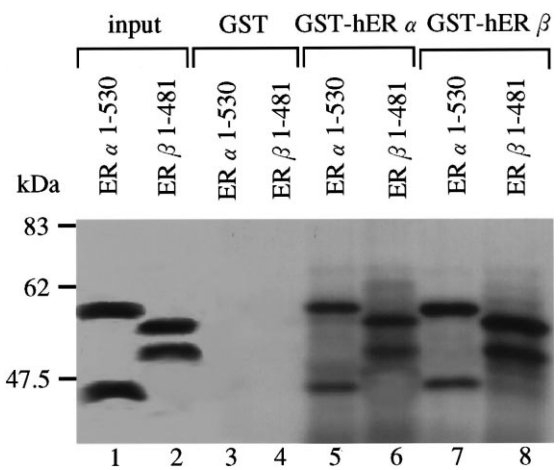

Fig. 4. GST pull-down assay. ERα1–530 and ERβ1–481 proteins were synthesized in vitro using the TnT-coupled reticulocyte lysate system (Promega), shown in lanes 1 and 2 at the amount of 10% of input. After binding of GST, GST-hERα and GST-hERβ proteins to glutathione beads, 15 μl of the suspension was incubated with 1–2 μl of the appropriate ^{35}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.

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).

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 ERα and ERβ 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, ERα1–530 and ERβ1–481, against wild-type ERα and ERβ. Both mutants were found to inhibit the transactivation by ERα as well as by ERβ. 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 ERα1–530 retains the region important for ER dimerization (500–520 aa) [17] and ERβ1–481 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 ERα was not soluble, we here utilized GST fusion protein with N-terminal truncated ERα. The protein interactions were demonstrated clearly as shown in Fig. 4. Thus, it was shown that both ERα1–530 and ERβ1–481 can act by heterodimerization with wild-type ERα as well as ERβ, at least. The sizes of the ERβ1–481 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 ERα1–530, the 58 kDa protein was translated in vitro as well as the 47 kDa protein, both of which were detectable by the anti-ERα monoclonal antibody D547 [18,19] (data not shown). The 47 kDa protein might be translated from downstream ATGs of ERα1–530 or its degradation product.

To characterize the cross-talk between ERα and ERβ in detail, it is also necessary to investigate the distinct functions of ERα and ERβ. The homology of the N-terminal A/B domains including the activation function 1 (AF-1) of ERα and ERβ is only 30% [11], which might be responsible for the selectivity of the tamoxifen antagonism only observed in ERα [12]. Moreover, E_2-dependent transactivation properties of ERα and ERβ on AP1 were recently reported to be in opposite modes [20]. The specificity of the transactivational regulation between ERα and ERβ 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 ERα and ERβ 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 ERα and ERβ mutants which block the function of both ERα and ERβ at the transactivation level. Moreover, we have shown evidence for the first time for the cross-interaction of these mutants with wild-type ERα and ERβ. 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 HEG0 and Dr. Greene for the gift of D547 monoclonal antibody. S.O. was supported by a JSPS Research Fellowship for Young Scientists.

References

[1] Evans, R.M. (1988) Science 240, 889–895.
[2] Green, S. and Chambon, P. (1988) Trends Genet. 4, 309–314.
[3] Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S. and Gustafsson, J.Å. (1996) Proc. Natl. Acad. Sci. USA 93, 5925–5930.
[4] Messelman, S., Polman, J. and Dijkema, R. (1996) FEBS Lett. 392, 49–53.
[5] Tremblay, G.B., Tremblay, A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Labrie, F. and Giguere, V. (1997) Mol. Endocrinol. 11, 353–365.
[6] Cowley, S.M., Hoare, S., Mosselman, S. and Parker, M.G. (1997) J. Biol. Chem. 272, 19858–19862.
[7] Pettersson, K., Grandien, K., Kuiper, G.G. and Gustafsson, J.Å. (1997) Mol. Endocrinol. 11, 1486–1496.
[8] Ince, B.A., Zhuang, Y., Wrenn, C.K., Shapiro, D.J. and Katzenellenbogen, B.S. (1993) J. Biol. Chem. 268, 14026–14032.
[9] Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I. and Chambon, P. (1989) EMBO J. 8, 1981–1986.
[10] Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Gene 108, 193–199.
[11] Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y. and Muramatsu, M. (1998) Biochem. Biophys. Res. Commun. (in press).
[12] Watanabe, T., Inoue, S., Ogawa, S., Ishii, Y., Hiroi, H., Ikeda, K., Orimo, A. and Muramatsu, M. (1997) Biochem. Biophys. Res. Commun. 236, 140–145.
[13] Inoue, S., Kondo, S., Hashimoto, M., Kondo, T. and Muramatsu, M. (1991) Nucleic Acids Res. 19, 4091–4096.
[14] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–467.
[15] Inoue, S., Orimo, A., Hosoi, T., Kondo, S., Toyoshima, H., Kondo, T., Ikegami, A., Ouchi, Y., Orimo, H. and Muramatsu, M. (1993) Proc. Natl. Acad. Sci. USA 90, 11117–11121.
[16] Schodin, D.J., Zhuang, Y., Shapiro, D.J. and Katzenellenbogen, B.S. (1995) J. Biol. Chem. 270, 31163–31171.
[17] Fawell, S.E., Lees, J.A., White, R. and Parker, M.G. (1990) Cell 60, 953–962.
[18] Greene, G.L., Nolan, C., Engler, J.P. and Jensen, E.V. (1980) Proc. Natl. Acad. Sci. USA 77, 5115–5119.
[19] Greene, G.L., Sobel, N.B., King, W.J. and Jensen, E.V. (1984) J. Steroid Biochem. 20, 51–56.
[20] Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J.Å., Kushner, P.J. and Scanlan, T.S. (1997) Science 277, 1508–1510.