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The Complete Primary Structure of Human Estrogen Receptor β (hERβ) and Its Heterodimerization with ER α *in Vivo* and *in Vitro*

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Human estrogen receptor β (hERβ) cDNA that encodes the full-length amino acid sequence has been isolated from testis poly(A)$^+$ RNA with the combination of cDNA screening and reverse transcription-PCR. It is composed of a 1590-bp open reading frame and a segment of the 5′- and 3′-untranslated region (UTR) and encodes an additional 53 amino acids in the N-terminal region compared with the previously reported one. Protein interaction between ERα and ERβ was demonstrated *in vitro* by GST pull-down assay and *in vivo* by immunoprecipitation. Thus, this study indicates that ERα and ERβ can interact *in vivo,* cross-signaling each other.

Estrogen receptor (ER) is a member of the nuclear receptor superfamily and plays a crucial role in the sexual development and the reproductive cycle in females (1-3). In addition, it is found in various tissues and cells of both sexes including brain, bones and artery, and implicated in the physiology of these organs (4,5).

Gene targeting experiments have shown that the mice homozygous for the disrupted gene (ERKO) show serious underdevelopment of reproductive organs (6). However, they are still viable and the uterus from ERKO females still have some estrogen (E_2) binding activity (7), suggesting the possibility of the existence of another type of estrogen receptor.

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Abbreviations used: ER, estrogen receptor; ERE, estrogen response element; DBD, DNA binding domain; RT-PCR, reverse transcription PCR; UTR, untranslated region; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; LBD, ligand binding domain; AF-2, activation function 2.

Although many nuclear receptors activated by non-steroid ligands, such as RAR, RXR, and TR have multiple subtypes and isoforms (8-10), few such instances were reported in the steroid hormone receptor. Recently, cDNAs of another receptor of estrogen, named ERβ, have been cloned from rat (11), human (12) and mouse (13). They all have the DNA binding domain which is almost identical to the homologous ERα, implying that both ERα and ERβ might share the same DNA response element. More recently, the heterodimer complex of ERα and ERβ has been demonstrated, suggesting their cross-talking possibilities (14,15).

In this study, we have isolated a human cDNA clone which appears to represent the complete sequence of ERβ. We have also demonstrated the molecular interaction of ERα and ERβ *in vivo* as well as *in vitro.*

MATERIALS AND METHODS

Plasmid construction. The ERα cDNA originated from HEG0 (16) was cloned into the pCXN2 expression vector (17) to construct pCXN2-hERα. The ERα cDNA encoding amino acids from 117 to 595 was cloned into pGEX4T-2 (Pharmacia) to construct GST-hERα. The RT-PCR product of ERβ cDNA (nucleotides from 1 to 1740) was cloned into pCXN2 and into pGEX4T-2 (Pharmacia) to construct pCXN2-hERβ, and GST-hERβ, respectively. The PCR amplified products of HA-tagged (18) ERβ were cloned into pCXN2 to construct pCXN2-HA-hERβ. Construction of ERE-GCAT was described elsewhere (19). All constructs were verified by sequencing.

Screening of human cDNA library. A human testis λZAPII cDNA library (5.0×10^5 plaques) constructed in pBK-CMV (Stratagene) was screened with a ^{32}P-labeled DBD fragment of the rat ERα cDNA encoding from 177 a.a. to 281 a.a.. The plaque-transferred filters were hybridized with the probe for 18 h at 63°C in 5×SSC, 0.5% (w/v) blocking agent (Amersham). The filters were washed for 15 min at room temperature in 2×SSC and 0.1% SDS twice, and then exposed to X-ray film. Further screening was repeated until single positive signal was obtained.

DNA sequence and analysis. The nucleotide sequences were determined by repeating sequencing of both strands of alkaline-dena-

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tured plasmid DNA using the BcaBest sequencing kit (TaKaRa Co.). The obtained DNA sequence was compiled and analyzed using DNASIS computer programs (Hitachi Co.).

Reverse-transcription PCR (RT-PCR). The oligonucleotide primer pairs of 20 nucleotides were designed based on the 5′-extended clone of ERβ derived from cDNA screening (5′-GTTGACAGCCATTATACTTG-3′) and 3′UTR sequence of published ERβ cDNA (12) (5′-ACGATTCAGCTTGTGACCTC-3′). cDNA was synthesized from 0.1 μg of human testis poly(A)$^+$RNA (Clontech) using random 9 mers and AMV reverse transcriptase (TaKaRa Co.). Subsequent PCR amplification was carried out by the RNA PCR kit (AMV) (TaKaRa Co.) for 30 cycles using an annealing temperature of 55°C in a Perkin Elmer thermalcycler (Perkin Elmer Cetus, Norwalk, CT). The specific PCR products were subcloned into pCRII vector (Invitrogen) and sequenced.

Cell transfection and whole cell extracts preparation. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 10% dextran-coated charcoal-stripped fetal calf serum (20). The 5×10^5 cells in 10 cm petri dishes were transfected with a total 20 μg of plasmids by using calcium phosphate (21). 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).

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, solublized, 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 floride). 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.

Immunoprecipitation. COS-7 cells were cotransfected with expression plasmids encoding ERα and HA-tagged ERβ. Cell extracts containing 100-200 μg total protein were incubated with an anti-ERα monoclonal antibody D547 (22-24) or an anti-HA monoclonal antibody 12CA5 (Boehringer Co.) on ice for 1 h, in buffer containing 20 mM HEPES, pH 7.8, 50 mM KCl, 10% glycerol, 1 mM DTT and 5 μg/ml phenylmethylsulfonyl fluoride, followed by incubation with protein G for further 3 h. The immunoprecipitates were pelleted by centrifugation at 12,000×g for 5 min, released into 20 μl of 1×SDS/PAGE buffer by boiling for 5 min, and analyzed on immunoblots using anti-ERα monoclonal antibody H222 (24) or 12CA5 using the chemiluminescence-based ECL detection system (Amersham) according to the manufacturer's instruction.

RESULTS

Cloning and identification of the full-length ERβ. A 5′-extended clone of ERβ derived from cDNA screening mentioned in Materials and Methods contained an extra 5′-stretch in addition to the human ERβ sequence which had been reported previously (12). Then, RT-PCR was carried out with the human testis poly(A)$^+$ RNA using the PCR primer pair based on the 5′-extended sequence of ERβ and the 3′-UTR sequence of published ERβ (12). The amplified product was detected at the size of 1740 bp, and then subcloned and sequenced (see DNA Data Bank of Japan accession no. AB006590). It was composed of a 1590 bp open reading frame and segments of the 5′- and 3′-UTR, encoding additional 53 amino acids in the N-terminal region (Fig. 1A) compared with the published sequence (12). The predicted ERβ protein consists of 530 amino acids, with a calculated relative molecular mass (Mr) of 59.2 KDa counted from ATG codon of the nucleotide 99 preceded by an in-frame stop codon at nucleotide 33. Thus, we regard this to be the full-length open reading frame of ERβ. Northern blot analysis showed 7.5 kb ERβ transcript both in testis and ovary (data not shown). Comparison of the structures among ERα and ERβ is shown in Figure 1B.

Interaction between ERα and ERβ in vitro and in vivo. The GST-fusion ERβ protein was produced at the size of 87 kDa. *In vitro* translated products of ERα consisted of two proteins of 66 kDa and 55 kDa and both of them were pulled down using the GST-fusion ERβ protein (Fig. 2, lanes 1, 5). As for ERβ products, 60 kDa and 57 kDa proteins were detected and both of them were pulled down using GST-fusion ERβ protein (Fig. 2, lanes 2, 6). *In vitro* translated ERα and ERβ were not pulled down with the GST protein alone (Fig. 2, lanes 3, 4).

To see if these subtype molecules actually interact with each other *in vivo,* the whole cell extracts of COS-7 cells cotransfected with ERα and/or HA-ERβ were prepared for the immunoprecipitation assay (Fig. 3). No band was detected by anti-human ERα monoclonal antibody H222 nor by anti-HA monoclonal antibody 12CA5 in the extracts of COS-7 cells without transfection (lanes 1, 9). When the extracts were immunoprecipitated by either anti-ERα monoclonal antibody D547 or 12CA5, no band was immunodetected either (lanes 3, 6, 11, 14). When COS-7 cell extracts transfected with pCXN2-hERα were immunoprecipitated by D547, ERα was immunodetected by H222 but ERβ was not by 12CA5 (lanes 4, 12). When the cell extracts transfected with pCXN2-HA-ERβ were immunoprecipitated, HA-ERβ was immunodetected by 12CA5 but not by H222 (lanes 7, 15). When pCXN2-hERα and pCXN2-HA-ERβ were cotransfected in COS-7 cells, both ERα and HA-ERβ bands were detected by H222 and 12CA5, respectively (lanes 2, 10). When the extracts were immunoprecipitated by D547, the precipitating band was identified by both H222 and 12CA5 (lanes 5, 13). In a reciprocal experiment, 12CA5 immunoprecipitate was detectable by H222 and by 12CA5 as well (lane 8, 16). Together, these results clearly indicate that ERα and ERβ associate each other under certain conditions *in vivo.*

DISCUSSION

Here, we isolated and identified for the first time the full-length human ERβ. Three in-frame ATG codons of

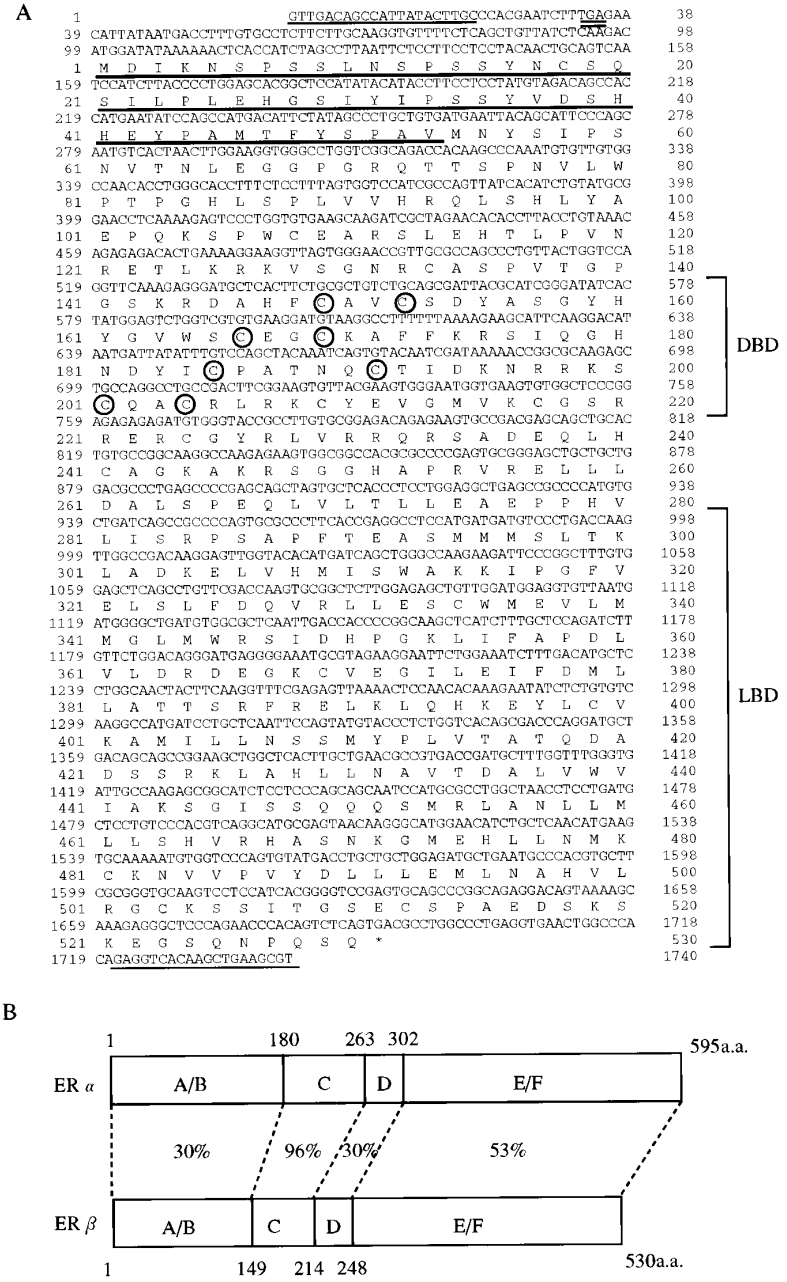

FIG. 1. Structures of hERβ. (A) Nucleotides and deduced amino acid sequences of full-length cDNA of hERβ. Single-letter notation is used for the amino acids. The in-frame stop codon at nucleotide 33 is shown as double underlined. The primer pairs used for RT-PCR and additional 53-amino-acid sequence compared with the published one are also underlined. The cysteine residues involved in the zinc-finger structures of the DNA-binding domain are circled. (B) Comparison of the structures between ERα and ERβ. The functional A to E/F domains are schematically represented, with the numbers of amino acid residues indicated. Percentage of amino acid identity is depicted.

ERβ are located at nucleotide 99, 234 and 258, preceded by an in-frame stop codon at nucleotide 33, which suggests that they are possible start codons. Among these three ATG codons, the 1st and the 2nd ATG codons conform to the Kozak consensus sequence (25). The longest open reading frame of ERβ encodes proteins of 530 amino acid residues with a calculated molecular weight of 59.2 kDa (calculated from the 1st methionine). The sizes of the ERβ protein translated *in vitro* were approximately 60 kDa and 57 kDa (data not shown), suggesting its translational initiation from both the 1st ATG and its downstream ATGs. Since the published ERβ cDNA lacks an in-frame stop codon further upstream, this 1st ATG codon described here may be the start codon for ERβ. This 5′-extended region encoded the additional 53 amino acids in its A/B do-

main compared with the published ERβ sequence. In view of the general conservation of the steroid receptors, the sizes of ERβ of rat and mouse might be longer than previously reported molecules (11,13).

During the preparation of this manuscript, the heterodimerization between ERα and ERβ was demonstrated by other groups (14,15). Cowley et al. showed an ERα/ERβ heterodimer by using gel-shift assay, which might enhance the mutual transcriptional activity additively rather than synergestically. Pettersson et al. also showed the complex by GST-pull down assay with GST-ERβ and by mammalian two-hybrid system (14,15). In our experiments, both ERα and ERβ were also pulled-down *in vitro* by GST fusion full-length hERβ (Fig. 2). Moreover, we showed that ERα and ERβ were co-immunoprecipitated *in vivo* (Fig. 3), thus clearly indicating the heterodimerization *in vivo*. This may suggest their cross-signaling in the cell.

To establish the cross-signaling between ERα and ERβ in detail, it is also necessary to investigate the distinct functions of ERα and ERβ. We previously reported that the agonistic effect of tamoxifen was selectively observed in ERα, but not in ERβ (19), which might be due to the difference of the activation function 1 (AF-1) region essential for the tamoxifen antagonism. The homology of N-terminal A/B domain including AF-1 between ERα and ERβ is only 30%. In addition, it was recently demonstrated that E2 inhibited the transactivation by ERβ at AP1 sites, which was in contrast to the activation by ERα (26). Furthermore, a cofactor SRC-1 selectively enhanced the ligand-independent transactivation of mouse ERβ (13). These findings suggest the possibility that the distinct transactivational mechanisms are operative between ERα and ERβ due to their different AF-1 and AF-2 functions. Heterodimerization of these receptors might modulate mutual function in target tissues and transactivation yet to be elucidated. As a result, it might explain some aspects of the various physiological phenotypes by estrogen. In addition, the engineering and the analyses of the ERβ and ERα/β knockout mice will provide us with more insights into the diverse estrogen action *in vivo*.

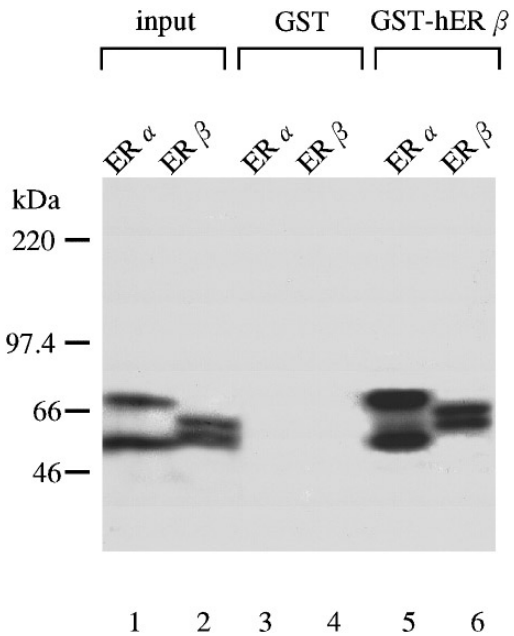

FIG. 2. Interaction between ERα and ERβ *in vitro* by GST-pull down assay. ERα and ERβ proteins were synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega), shown in lanes 1 and 2 at the amount of 10% of input, respectively. After binding of GST and GST-ERβ proteins to glutathione 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.

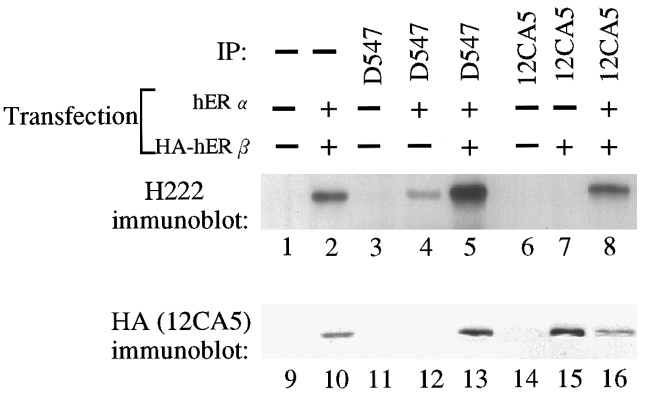

FIG. 3. Co-immunoprecipitation of ectopic HA-tagged ERβ with ERα *in vivo*. Anti-ERα monoclonal antibody D547 (lanes 3-5, 11-13) and anti-HA monoclonal antibody (12CA5) (lanes 6-8, 14-16) were used for immunoprecipitation. The upper and the lower panels indicate the immunoblots by anti-ERα monoclonal antibody H222 (lanes 1-8) and 12CA5 (lanes 9-16), respectively.

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