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A Novel Isoform of Rat Estrogen Receptor Beta with 18 Amino Acid Insertion in the Ligand Binding Domain as a Putative Dominant Negative Regulator of Estrogen Action

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A novel isoform of rat estrogen receptor (ER) beta, ERβ2, which is a putative alternative splicing product of the reported ERβ (ERβ1) has been identified. Rat ERβ2 cDNA contains an additional in-frame 54 base pair insertion in the ligand binding domain of ERβ1, which generates an 18 amino acid residue insertion. Northern blot and RT-PCR analyses revealed that ERβ2 coexists with ERα and ERβ1 in all tissues examined including brain, lung, liver, kidney, fat, bone, uterus, prostate, and ovary. The insertion caused loss of ligand binding activity of ERβ2, whereas the ability to bind the palindromic estrogen response element (ERE) was retained. In an ERE-containing luciferase reporter gene assay using COS-1 cells, ERβ2 failed to activate estrogen-dependent transcription. Furthermore, ERβ2 dose dependently suppressed the ERα- and ERβ1-mediated transcriptional activation. These results suggest that rat ERβ2 functions as a negative regulator of estrogen action.

Since the initial cDNA cloning of nuclear steroid hormone receptor proteins in the 1980's, the superfamily of nuclear receptors has grown rapidly. This superfamily is characterized structurally by six discrete domains (A-F) and functionally by increasing transcriptional activity (1-4). Among the members of the superfamily, the DBD (C domain) and the LBD (E/F domain) are well-conserved evolutionarily (5, 6). There are several subtypes which are similar in sequence and share the same ligands, *e.g.* thyroid hormone receptors (TR), retinoic acid receptors (RAR) and retinoid X receptors (RXR), however, the first and so far only subtype of a sex steroid receptor, ERβ, was not found until 1996 (7-9). The expression of rat (r) ERβ mRNA was dominant in ovary and prostate. ERβ was thought to play unique physiological roles compared to classical ER, ERα (10-12), because the expression pattern and its regulation of ERβ were different from those of ERα (13, 14). In addition, Northern blot analysis of ERβ revealed multiple transcripts which suggested alternative splicing products (8, 9, 14). Concerning human (h) ERα, several splicing variants which lack one or two exons among eight coding exons (15) have been found in tumor cells, brain, bone and vascular smooth muscle cells (16-20). Functions of these variants have been partially characterized: the variant lacking exon 3 (Δ3) in the DBD showed dominant negative effect and no ERE binding (16); Δ7 and Δ4/5 had dominant negative action (16, 20); Δ5 was a constitutive active form (16). The physiological roles of these variants have not been understood well and the molecular mechanism underlying their synthesis remains to be elucidated.

In the present study, a novel isoform of ERβ (rERβ2) was identified which appears to be an alternative splicing product of rERβ. rERβ2 contains an 18 amino acid insertion in the LBD of rERβ reported before (designated rERβ1). Analysis of this variant suggests that rERβ2 may act as a dominant negative regulator for estrogen actions.

MATERIALS AND METHODS

RT-PCR, DNA sequencing, and Northern blot analysis. First strand cDNA was synthesized using Advantage RT-for PCR kit (Clontech) and poly A^+ RNA of rat ovary, prostate, brain, lung, liver, kidney, fat, bone, and uterus. The oligonucleotide primers to amplify the rERβ (Genbank, U57439) cDNA were as follows: A, cttcctcctacg-

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Abbreviations: ER, estrogen receptor; RT-PCR, Reverse transcriptase polymerase chain reaction; bp, base pair; ERE, estrogen response element; DBD, DNA binding domain; LBD, ligand binding domain; GST, glutathione-*S*-transferase; E_2, 17β-estradiol.

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/Genbank nucleotide sequence databases with Accession Number AB012721.

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tagacaaccg; B, accatctctgtagtctgtccgc; C, gagctcagcctgttggacc; D, ggccttcacacagagatactcc. The primers for rERα (Genbank, Y00102) were as follows: E, ggctacgtcaagtcgattcc; F, atcttgtccaggactcggtg. Oligonucleotides corresponding to the 54 base pair insertion were as follows: G, agcactcttcatctgcgcaacgtgccagtga; H, actcttcatctgcgcaacg; I, cgcagatgaagagtgctgc. The ORF of ERβ was amplified using an LA-PCR kit (Takara, Kyoto, Japan) under the following conditions: denaturing at 98°C for 10 seconds and extension at 68°C for 5 minutes, repeated for 25 cycles. The PCR products were subcloned into pCR2.1 vector (Invitrogen). The sequence was determined on both strand at least twice on 377A DNA sequencer (Perkin-Elmer). PCR for the LBD of rERs was done under the following conditions: denaturing at 94°C for 1 minute, annealing at 60°C 50 seconds, extension at 72°C for 2 minutes, repeated for 30 cycles. Genomic PCR was done using an LA-PCR kit under the following conditions: denaturing at 98°C for 10 seconds and extension at 68°C for 15 minutes, repeated for 25 cycles.

Poly A^+ RNA (2 μg each lane) was separated electrophoretically and blotted onto nylon membranes (Pall Biosupport) using standard methods (21). The rERα and rERβ1 cDNAs were labeled with [^{32}P]-α-dCTP (Amersham) by random priming (21). The oligonucleotide G was 5′ end-labeled with [^{32}P]- γ-ATP (Amersham). Prehybridization and hybridization were done as described (21) using 2 x 10^6 cpm/ml denatured probe. Washing was done as follows: for cDNA probes, 0.2 x SSPE/0.03% sodium pyrophosphate/0.1% SDS at 55°C for 30 minutes; for oligonucleotide G, 2 x SSPE/0.03% sodium pyrophosphate/0.1% SDS at 42°C for 30 minutes. The membrane was directly exposed to the imaging plate of a BAS 2000 image analyzer (Fuji Film, Tokyo, Japan).

Functional study of rat ERβ2. The rERβ expression vectors were constructed by ligating coding sequences into the Eco RI site of pSG5 (Stratagene). Plasmid pCH110, a β-galactosidase (β-gal) reporter was from Pharmacia. Rabbit β-globin TATA box (-40 to +10) was introduced into the upstream region of the luciferase (Luc) gene of the pGL2-basic vector (Promega). Palindromic ERE was fused upstream of TATA box to construct ERE-Luc vectors. GST fusion proteins were generated by subcloning fragments of the rER cDNAs corresponding to the LBD into the pGEX-2T expression vector (Pharmacia).

GST/ER fusion proteins were expressed in bacteria as followed. A 400 ml *E. coli* culture LB media was allowed to grow for 6 hours at 27°C after 2.5 mM isopropyl-β-D-thiogalactopyranoside induction. Harvested cells were resuspended in ice-cold buffer A (20 mM Tris-HCl pH 7.5/0.15 M NaCl/10% glycerol (v/v)/0.055% 2-mercaptoethanol/1 mM EDTA/0.1 mM EGTA/0.5 mM PMSF), and then sonicated at 150 W for 10 minutes. GST/ER fusion protein was adsorbed to glutathione-Sepharose 4B, collected by centrifugation, and washed in buffer B (0.5% NP-40 containing buffer A) four times.

For binding studies, the binding mixture contained 0.375 μg GST/ER adsorbed to glutathione-Sepharose 4B and 50 μl of [6,7-^{3}H(N)]-estradiol (Du Pont) in ethanol and its volume was adjusted to 300 μl using TEG buffer (10 mM Tris-HCl pH 7.4/1.5 mM EDTA/10% glycerol). A 1000-fold excess of cold E_2 was added to the mixture to estimate non-specific binding. The mixture was incubated at 4°C overnight with gentle shaking and passed through glass filter to trap labeled estradiol bound to GST/ER. The glass filter was washed with 10 mM Tris-HCl (pH 7.4) and radioactivity was determined by liquid scintillation counting.

Estrogen-dependent transactivation via ER was investigated by Luc reporter assay. For transfection, COS-1 cells were seeded in 60 mm dishes in phenol red-free DMEM (GIBCO BRL) supplemented with 10% charcoal dextran-treated fetal bovine serum. At 50-80% confluency, cells were transfected using LIPOFECTAMINE (GIBCO BRL) following manufacturer's protocols. Expression plasmids used were as follows: 4 μg of total DNA containing 2 μg of reporter, 0.6 μg of pCH110, 0.2-0.6 μg receptor, and 0.8-1.2 μg of Bluescribe $M13^+$ carrier DNA. After 20-24 hours, cells were washed and replaced with fresh medium containing either 10 nM E_2 or no E_2. After 20-24 hours, the cells were lysed to determine Luc and β-gal activity.

For DNA binding studies, nuclear extracts of COS-1 cells overexpressing rERβ2 were prepared following Dignam's methods (22). Oligonucleotide probes (ERE: agctcaaggtcacagtgacctcaagct, EREm: agctcaagtgcacagtggtctcaagct) were chemically synthesized as double-strands, annealed, and ^{32}P-labeled as described before (23, 24). The binding reaction was started by adding labeled oligonucleotide (2 x 10^4 cpm) and incubated at 25°C for 15 minutes in binding buffer (10 mM Hepes-KOH pH 7.8/25 mM KCl/1 mM $MgCl_2$/2.5 mM DTT/10% glycerol) containing nuclear extract (1-5 μg of protein). The reaction mixture was analyzed on a 5% polyacrylamide gel as described before (24).

RESULTS

Identification of the rat ERβ isoform. A 1.6 kb fragment was amplified from rat ovary cDNA using the A and B primer set, which bracket the rERβ ORF (Fig. 1A). An additional 0.3 kb fragment was amplified alone with an expected size fragment (246 bp) from the same cDNA using primers C and D which were designed to amplify the LBD of ERβ (Fig. 1B, lane 2). On the other hand, only the expected size fragment of the LBD of ERα was amplified (159 bp, Fig. 1B, lane 1). The RT-PCR products shown in Figs. 1A and 1B were subcloned and sequenced. The 300 bp band (Fig. 1B lane 2) turned out to be a novel ERβ cDNA fragment containing an in-frame 54 bp insertion in the LBD. This was designated as the ERβ2 to distinguish it from the previously reported ERβ, ERβ1. The 1.6 kb band in Fig. 1A also contained at least two cDNA fragments, one is ERβ1 the other is ERβ2 judging from the sequencing results of subcloned cDNAs.

Results of Northern blot analysis are presented in Fig. 1C. Multiple signals were detected using the full-length cDNA of ERβ1 (panel a) as reported before (8, 9, 13), showing the most intense signal at 7.5 kb and additional signals at 10 kb, 4 kb, and 2 kb. In panel b, a 7.5 kb band also hybridized with oligonucleotide G, corresponding to the sequence of 54 bp insertion. Although additional weak signals at 4.5 kb and 1.5 kb were seen in panel b, their sizes were not coincident with those of any signals shown in panel a, suggesting that they were non-specific signals. Signals hybridizing with oligonucleotide G were detected only in rat prostatic, ovarian and uterine tissues, probably due to lower expression of these transcripts (Fig. 1C, panel b; additional data not shown). The expression patterns of ERα in these tissues are different from those of ERβ as shown in panel c. As shown in panels e and f in Fig. 1C, ERβ1 cDNA hybridized to both ERβ1 and ERβ2 cDNAs, whereas oligonucleotide G hybridized to only ERβ2 cDNA.

RT-PCR study demonstrated that ERβ2 was expressed in all rat tissues examined, including brain, lung, liver, kidney, fat, bone, uterus, prostate, ovary, both with ERα and ERβ1 (Fig. 1D). The ERβ2 signal in the kidney was faint in this experiment. Our results

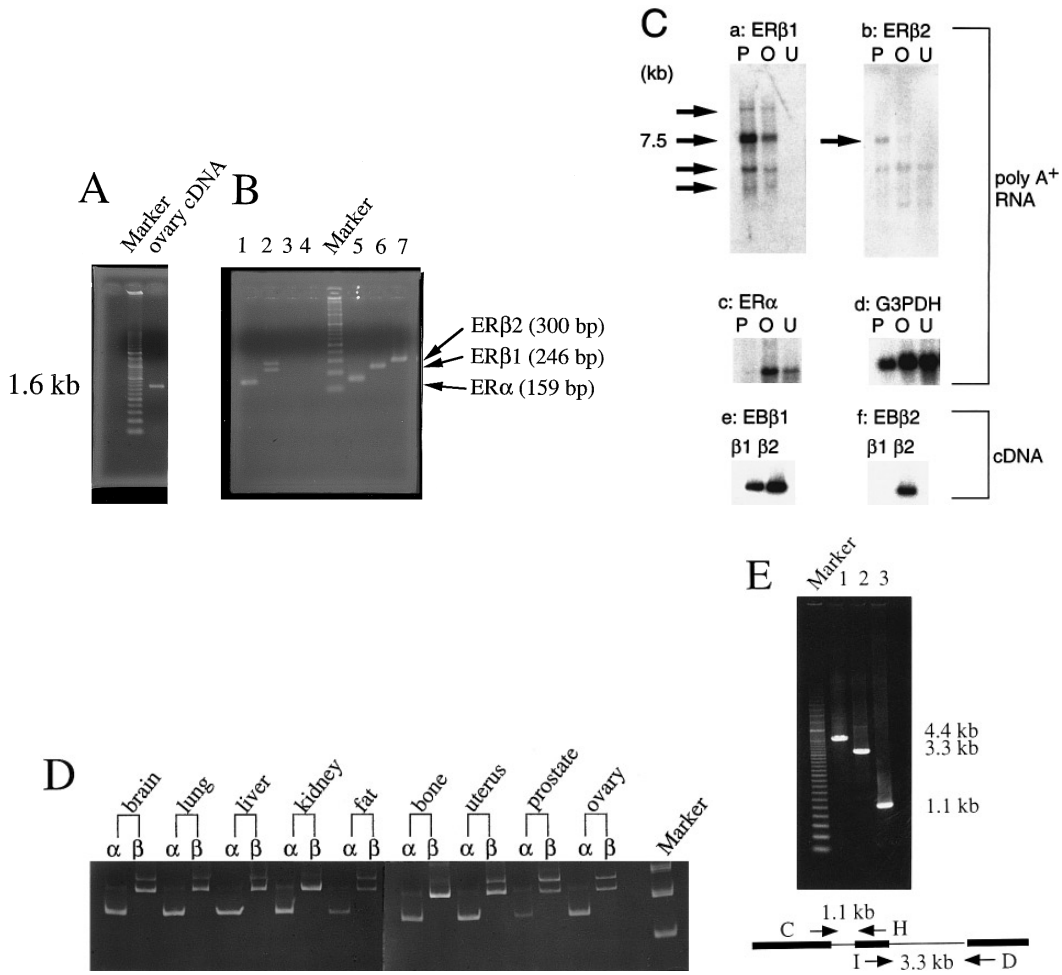

FIG. 1. Expression of the rat ERβ isoform. (A) RT-PCR for full-length rat ERβ from ovary cDNA using primers A and B. Molecular weight markers define a ladder of 200 bp steps. The PCR products were separated on a 0.8% agarose gel. (B) RT-PCR for the LBD of rat ERs. ERα primers E and F were used in lanes 1 (ovary RNA), 3 (ovary RNA, reverse transcription negative) and 5 (ERα cDNA). ERβ primers C and D were used in lanes 2 (ovary RNA), 4 (ovary RNA, reverse transcription negative), 6 (ERβ1 cDNA), and 7 (ERβ2, cDNA). The PCR products were separated on a 2% agarose gel. Molecular weight markers define a ladder of 200 bp steps. (C) Northern blot analysis for rat ERs. Abbreviations for lanes indicate as follows: P, prostate; O, ovary; U, uterus. ^{32}P-Labeled probes used were as follows: (a) full-length rat ERβ1 cDNA, (b) oligonucleotide G, (c) full-length rat ERα cDNA, (d) human G3PDH control probe from Clontech. Rat ERβ cDNAs were electrophoresed and probed with full-length rat ERβ1 cDNA in e and oligonucleotide G in f. (D) RT-PCR for the LBD of rat ERs from various rat tissues. The primers used were C and D, and the PCR products were separated on a 5% polyacrylamide gel. Molecular weight markers indicate 100, 200, and 300 bp. (E) Genomic PCR for the LBD of ERβ2. Primers used were: C and D in lane 1, D and I in lane 2, C and H in lane 3, respectively. Molecular weight markers define a ladder of 200 bp steps. Schematic outlining where the primers are located in the rat genome is presented below the photograph.

shown in Figs. 1C and 1D reveal that ERβ2 coexists with ERα and ERβ1 in most tissues, but the expression ratio of these subtypes varies from tissue to tissue. The presence of the ERβ2 sequence in the rat genome was demonstrated by genomic PCR. As shown in Fig. 1E (lane 1), a 4.4 kb fragment was amplified using primers C and D from the rat genome as a template. This result indicates that the coding sequence of rat ERβ1 between primers C and D is interrupted by at least one intron. Next, combinations of primers D and I (lane 2) and primers C and H (lane 3) was tried. The results in Fig. 1E show that the additional 54 bp sequence was located 1.1 kb downstream of the primer C site and 3.3 kb upstream of the primer D site in the rat genome.

Sequence analysis of ERβ2. The additional 54 bp sequence generates an in-frame 18 amino acid insertion after Arg 319 in the LBD of ERβ2 (underlined in Fig. 2A). Amino acid sequences of ERαs and ERβs around the inserted 18 amino acid are well-conserved across different animal species (Fig. 2B). The location of the insertion exactly matches the border between exon 5 and 6 in the hERα (15). It is highly likely that exon/intron junctions are conserved among ER genes and that ERβ2 is an alternative splicing product of the

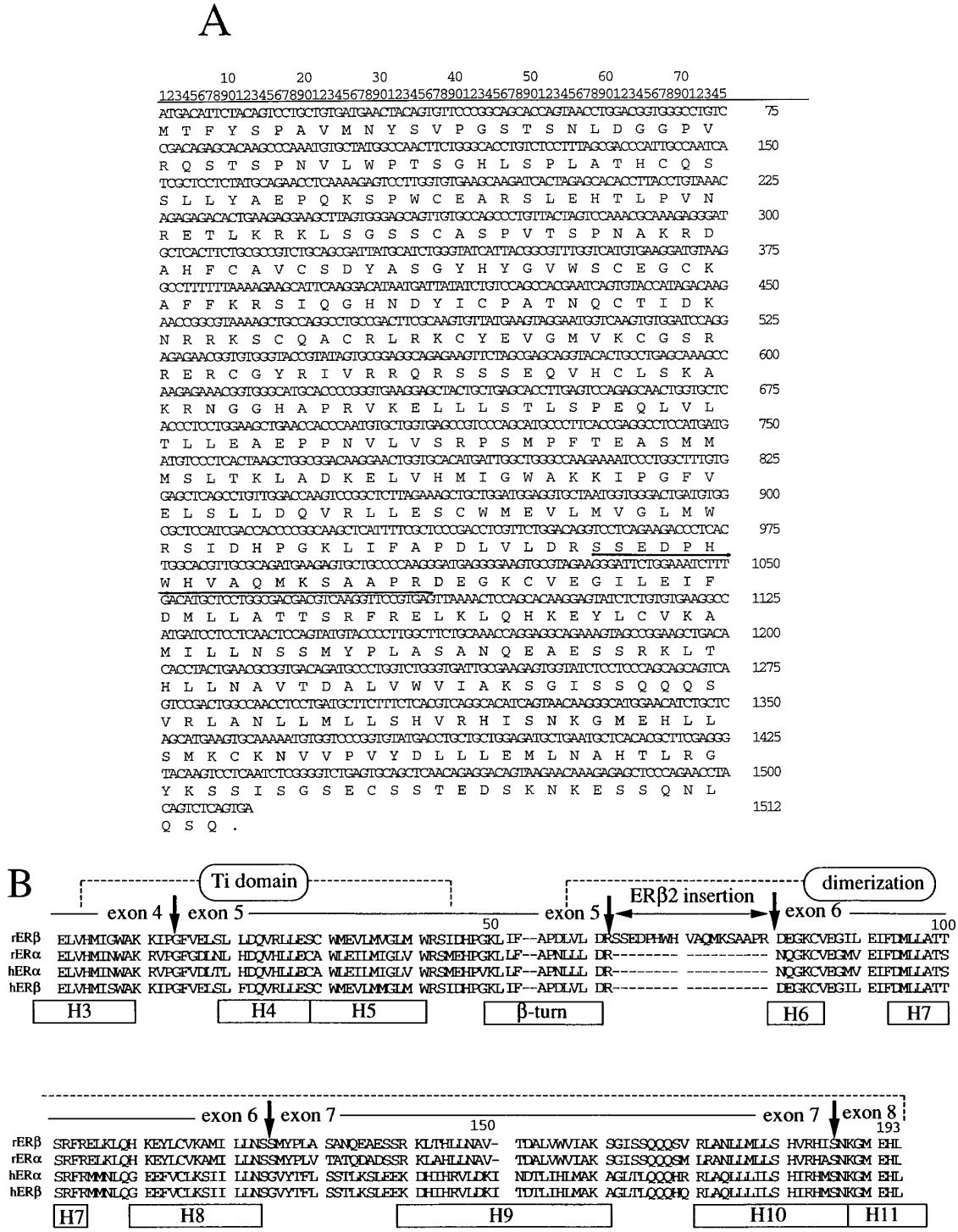

FIG. 2. Sequence of rat ERβ2. (A) Nucleotide and deduced amino acid sequence of rat ERβ2 cDNA. The sequence inserted into the rat ERβ1 cDNA is underlined. (B) Amino acid sequence alignment of ERs and steroid receptors from the Ti domain (6) to the central part of E domain devoted to dimerization (24). The Ti domain and the region devoted for dimerization process are indicated by dotted lines. Sequences corresponding to the secondary structure elements found in the LBD of RARγ (27) are indicated as follows: helices H3 to H11 and β-turn. Receptor sequences aligned are rERβ2, rERα (12), hERα (10, 11), hERβ (8).

ERβ gene. Structural analyses have demonstrated that the surrounding regions of the insert found in ERβ2 were topologically well-conserved among the nuclear receptor superfamily and constitute a ligand binding pocket demonstrated in RARγ, RXRα, TR, and ERα (25-29).

ERβ2 lacks ligand binding ability but inhibits estrogen-dependent transcriptional activation mediated by ERα and ERβ1. As shown in Fig. 3A, the GST/ERβ1 fusion protein revealed a *Kd* value of 0.32 nM on the competitive binding assay by measuring liberated [^{3}H]E_2 from the GST/ERβ1 after washing with buffer containing non-labeled E_2. These results are in a good agreement with previously reported results (*Kd* = 0.6 nM) (7) using *in vitro*-translated ERβ1 protein. In contrast, The GST/ERβ2 protein did not show any significant specific binding to [^{3}H]E_2.

Fig. 3B shows the comparison of estrogen-dependent transcriptional activation via rat ER subtypes using COS-1 cells. As expected from the results of ligand binding study, no significant estrogen-dependent transcriptional activation via ERβ2 was observed, whereas both ERα and ERβ1 mediated the estrogen-dependent transcriptional activation. Surprisingly, coexpression of ERβ2 with ERα showed significant suppression of the estrogen-dependent transcriptional activation via ERα. Similarly, ERβ2 suppressed the transcriptional activation via ERβ1. To further study the nature of ERβ2 molecule, a DNA binding assay was performed.

As depicted in Fig. 3C, ERβ2 showed specific binding to the palindromic ERE in spite of the 18 amino acid insertion in the LBD. Excess amount of cold ERE probe interfered with formation of ^{32}P-ERE/ERβ2 complex. EREm carries a mutation which abolishes ERα binding (24) and predictably showed no ERβ2 binding, but did not affect the formation of ^{32}P-ERE/ERβ2 complexes. These data demonstrate that ERβ2-ERE binding is specific.

DISCUSSION

The cDNA and protein product of a possible alternative splicing product of rERβ, designated rERβ2 has been cloned, purified and partially characterized. This protein has an 18 amino acid insertion in the LBD, which is the only difference from the previously reported rERβ protein. Amino acids that surround the inserted amino acid sequence, *i.e.*, H3-H5, β-turn, and H6-H12 are known to constitute a pocket for ligand binding in the case of RARγ, RXRα and TR (26-28), and it has recently been shown that the overall architecture of the ERα LBD is similar to that seen in the crystal structure of those nuclear receptor LBDs (29). This suggests that structural alteration of this area may perturb the structure of the pocket, thereby affecting ligand binding. Indeed, the insertion found in rERβ2 resulted in loss of ligand binding affinity (Fig. 3A). It is also consistent with the fact that ERβ2 does not induce estrogen-dependent transcriptional activation (Fig. 3B).

It is probable that the insertion may affect not only dimerization of rERβ2 but ERE binding efficiency as well. However, DNA binding assays demonstrated that

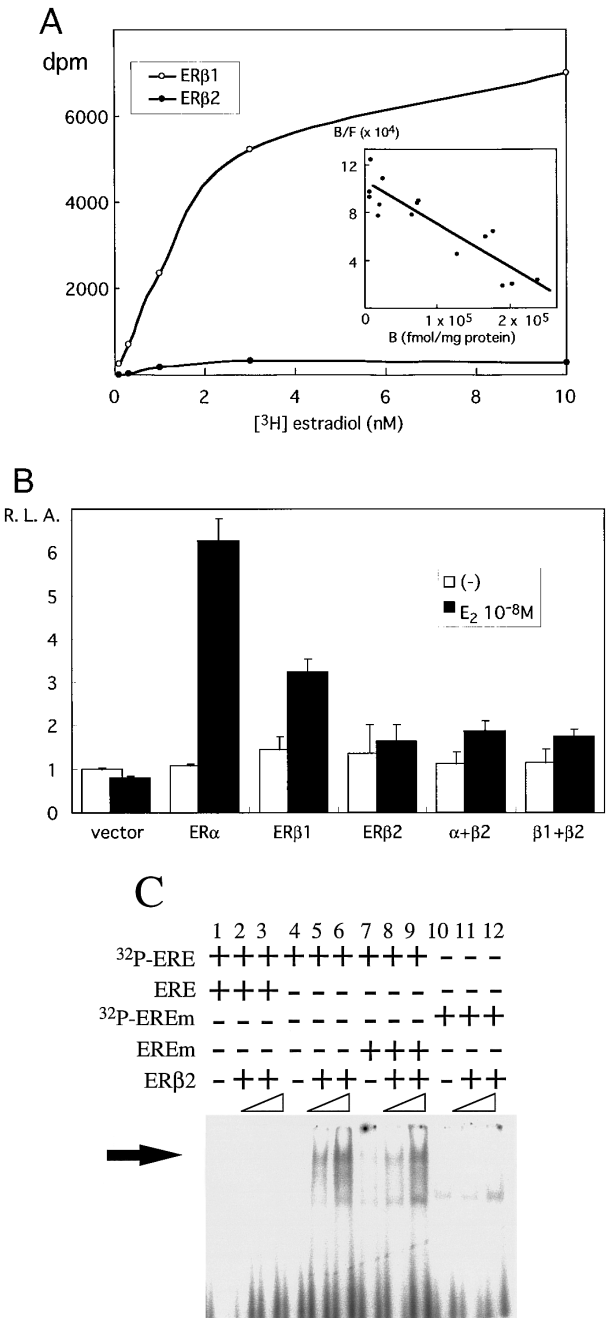

FIG. 3. Functional study of rat ERβ2. (A) E_2 binding properties of the LBD of rat ERβs expressed as GST fusion proteins. In the saturation plot, open circles (○) show the data for ERβ1 and closed circles (●) for ERβ2. Inset indicates the results of Scatchard plot for ERβ1 which shows the *Kd* value of 0.6 nM. Each point was determined in triplicate in each experiment. (B) E_2-dependent transcriptional activities of rat ERs. Ratio of receptors cotransfected was 1:1.5 for ERα vs ERβ1 and ERα vs ERβ2. Luc activity was normalized using β-gal activity and expressed as relative Luc activity (R. L. A.). Data show representative results from three independent experiments (the mean + S.E., n = 4). (C) DNA binding assay using the nuclear extracts obtained from the COS-1 cells overexpressing rat ERs. Proteins applied to the reaction were 0 μg (lane 1, 4, 7, 10), 1 μg (lane 2, 5, 8, 11), and 5 μg (lane 3, 6, 9, 12), respectively. Arrow indicates the ERβ2/DNA complex.

rERβ2 binds efficiently to ERE *in vitro* (Fig. 3C). Furthermore, rERβ2 inhibited E_2-dependent transcription activity of ERα and ERβ1 in a concentration dependent manner (Fig. 3C and data not shown), suggesting that this variant receptor may act as a dominant negative form of the ERs and thus may play an important role in the regulation of estrogen action. This possibility is also supported by the coexistence of ERβ2 with ERα and ERβ1 in all tissues examined in a various ratio (Figs. 1C and 1D).

The exact mechanisms by which rERβ2 causes inhibition of transcription activation mediated by ERα and ERβ are not clear at present. The most plausible mechanism is that rERβ2, as a monomer or homodimer, competes with heterodimers (27) or homodimers of ERα and ERβ in binding to ERE. It is also possible that ERβ2 may heterodimerize with ERα or ERβ and bind to ERE but somehow block the activation of transcription. Alternatively, availability or binding affinity of coactivators including SRC-1, CBP/p300, and RIP140 (30-33) to the receptor complexes may also be affected. These possibilities are subjects of future studies and are currently under investigation.

In summary, a possible splicing variant of ERβ, ERβ2, has been identified. This variant may function as a dominant negative regulator of estrogen action. These findings provide new insight into the regulatory mechanism of estrogen function. ERβ2 may prove useful in studying the structure and function of ERs.

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