ERβ: identification and characterization of a novel human estrogen receptor

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Abstract A novel estrogen receptor (hereinafter referred to as ERβ) was cloned using degenerate PCR primers. A comparison of the amino acid sequence of ERβ with the 'classical' ER (ERα) shows a high degree of conservation of the DNA-binding domain (96%), and of the ligand-binding domain (58%). In contrast, the A/B domain, the hinge region and the F-domain are not conserved. Northern blot analysis revealed that ERβ is expressed in human thymus, spleen, ovary and testis. Transient transfections of an ERβ expression construct together with an ERE-based reporter construct in CHO cells clearly demonstrated transactivation of ERβ by 17β-estradiol. In addition, the ERα antagonist ICI-164384 is a potent antagonist for ERβ as well. Interestingly, the level of transactivation by 17β-estradiol is higher for ERα than for ERβ, which may reflect suboptimal conditions for ERβ at the level of the ligand, responsive element or cellular context.

Key words: Estrogen receptor; 17β-Estradiol; Anti-estrogens; Transactivation; Human

1. Introduction

The estrogen receptor (ERα) is a member of the superfamily of nuclear receptors which are able to transduce extracellular signals (small lipophilic molecules) into transcriptional responses [1]. In general, nuclear receptors have a modular structure with six distinct regions, A–F. Of these, region C (corresponding to the DNA-binding domain) and region E (corresponding to the ligand-binding domain) are evolutionarily conserved [2]. Steroid hormone receptors are distinct from other nuclear receptors in a number of respects including the nature of their ligands, their association (in the unliganded state) with a repertoire of heat-shock proteins, and the fact that they bind to hormone response elements as homodimers [3]. Since the cloning of the ERα cDNA [4,5], a great deal of detailed knowledge regarding its mechanism of action has been obtained (see e.g. [6–8]). Basically, ERα dimerizes upon ligand binding and the homodimer then binds to estrogen response elements (EREs) in the transcriptional control regions of target genes. In addition, a number of pathways are now known to influence ER functioning: e.g. different conformations induced by different ligands, modifications of the receptor by phosphorylation and interactions of the receptor with other transcription factors [8–10]. In recent years, ERα has become an important target for the identification of compounds which may be effective in estrogen-dependent therapies such as hormone-replacement therapy and cancer.

Here, we report the identification and characterization of a novel human estrogen receptor (designated ERβ), which is highly homologous with the 'classical' ERα and has an overlapping but non-identical tissue distribution. We demonstrate that this novel receptor is functional in that it interacts with (anti-)estrogens and is able to modulate estrogen-responsive reporter gene expression.

2. Materials and methods

2.1. RT-PCR, RACE-PCR and primers

One microgram of total RNA (from EBV-stimulated human peripheral blood leukocytes) was reverse transcribed in a 20 μl reaction containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 4 mM $MgCl_2$, 1 mM dNTPs (Pharmacia), 100 pmol random hexanucleotides (Pharmacia), 30 units RNase inhibitor (Pharmacia) and 200 units M-MLV reverse transcriptase (Gibco BRL) at 37°C for 30 min followed by 5 min at 100°C. Two degenerate oligonucleotides were based on conserved regions of the DNA-binding domains (primer 1) and the ligand-binding domains (primer 2) of the human steroid hormone receptors. PCR reactions (100 μl) contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine (w/v), 3% DMSO, 1 μg of each primer and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer). The PCR protocol was: 4 min at 94°C, then 36 cycles (30 s 94°C, 30 s 45°C, 1 min 72°C) and finally 7 min at 72°C. All other PCR reactions were performed with an annealing temperature of 55°C and with 200 ng of each primer. Fragments of interest were cut out of a 1.5% agarose gel, reamplified and cloned using the TA-cloning kit (Invitrogen) and sequenced using a T7 DNA sequencing kit (Pharmacia).

RACE (rapid amplification of cDNA ends) PCR reactions were performed using a Marathon-ready kit (Clontech) with testis cDNA as template.

Primer 1: 5′-GGIGA(C/T)GA(A/G)GC(A/T)TCIGGITG(C/T)C-A(C/T)TA(C/T)\GG-3′
Primer 2: 5′-AAGCCTGG(C/G)A(C/T)IC(G/T)(C/T)TTIGCC-CAI(C/T)TIAT-3′
Primer 3: 5′-TGTTACGAAGTGGGAATGGTGA-3′
Primer 4: 5′-GGC(C/G)TCCAGCATCTCCAG(C/G)A(A/G)CA-G-3′
Primer 5: 5′-TCTTGTTCTGGACAGGGATG-3′
Primer 6: 5′-GGAAGCTGGCTCACTTGCTG-3′

2.2. Northern blot analysis

Human multiple tissue Northern blots (Clontech) were prehybridized for at least 30 min at 65°C in 0.5 M phosphate buffer pH 7.5 with 7% SDS. A ERβ-specific PCR fragment was generated using primer 2 in combination with primer 3. This probe was ^{32}P-labelled using a Decaprime kit (Ambion), denatured by boiling, added to the prehybridization solution and incubated overnight at 65°C. Washing conditions were: 3×SSC at room temperature, followed by 3×SSC at 65°C and then 1×SSC at 65°C.

2.3. Cloning of ERβ

Approximately 400000 recombinant phages of a human testis

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The nucleotide sequence of the cloned human ERβ has been assigned the accession number X99101.

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cDNA library in λgt11 (Clontech HL1010b) were screened. Replica filters (Hybond-N, Amersham) were made as described by the manufacturer and hybridized with the ERβ-specific probe (see Section 2.2). Filters were prehybridized in a solution containing 0.5 M phosphate buffer (pH 7.5) and 7% SDS at 65°C for at least 30 min. Probe-labelling and hybridization were as described in Section 2.2. Filters were washed in 0.5×SSC/0.1% SDS at 65°C. Positive clones were plaque-purified and fragments generated by PCR were cloned and sequenced. For verification of the nucleotide sequences the complete cDNA insert was generated using the proof-reading *Pfu* polymerase (Stratagene), cloned and both strands were sequenced.

In order to clone sequences downstream of exon 7, primer 4, based on the ERα AF-2 region was used in combination with ERβ-specific primer 5 (nucleotides 936–955 in Fig. 1A) and then nested with primer 6 (nucleotides 1130–1149 in Fig. 1A), using testis cDNA (Marathon-ready, Clontech) as template. A specific fragment was analyzed as described above. The remainder of the carboxy-terminus was cloned using RACE PCR reactions on the same testis cDNA. First primer 5 was used together with the AP1 primer provided in the kit, then a nested PCR was performed using primer 6 together with the CDS primer from the kit. Fragments that were obtained were cloned and sequenced.

2.4. Cell culture, transient transfections and transactivation assay

Chinese hamster ovary cells (CHO K1; CCL61) were cultured in phenol red-free M505 medium (a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Gibco) and nutrient medium F12 (Ham's F12, Gibco)) supplemented with 5% charcoal-treated bovine calf serum (Hyclone) and antibiotics. The ERβ reading frame was cloned in the mammalian expression vector pNGV1, a modification of pKCR [11] containing the SV40 early promoter and the IgG and MuLV enhancers. The reporter expression vector was based on the rat oxytocin gene regulatory region position −363/+16 [12] linked to the firefly luciferase gene. CHO cells (1×10^5) were seeded in 6-well Nunclon tissue culture plates and DNA (1 μg of both receptor and reporter constructs and 250 ng β-galactosidase vector in 250 μl Optimem, Gibco BRL) was mixed with an equal volume of lipofectin reagent (7 μl in 250 μl Optimem) and allowed to stand at room temperature for 15 min. This solution was mixed (1:1) with Optimem and 1 ml of this mixture was added to each well after washing the cells (serum-free M505). After a 5-h incubation the cells were washed (phenol red-free M505 with 5% charcoal-treated bovine calf serum) and incubated overnight at 37°C. After 24 h hormone was added to the medium. 17β-Estradiol (Org2317) was obtained from Organon, Oss, The Netherlands, ICI-164384 was kindly provided by Dr. A.E.

A

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GGCTATAGCCCTGCTGTGATGAATTACAGCATTCCCAGCAATGTCACTAACTTGGAAGGTGGGCCTGGTCGGCAGACCACAAGCCCAAATGTGTTGTGGCCAACACCTGGGCACCTTTCT  120
 G  Y  S  P  A  V  M  N  Y  S  I  P  S  N  V  T  N  L  E  G  G  P  G  R  Q  T  T  S  P  N  V  L  W  P  T  P  G  H  L  S    34

CCTTTAGTGGTCCATCGCCAGTTATCACATCTGTATGCGGAACCTCAAAAGAGTCCCTGGTGTGAAGCAAGATCGCTAGAACACACCTTACCTGTAAACAGAGAGACACTGAAAAGGAAG  240
 P  L  V  V  H  R  Q  L  S  H  L  Y  A  E  P  Q  K  S  P  W  C  E  A  R  S  L  E  H  T  L  P  V  N  R  E  T  L  K  R  K    74

GTTAGTGGGAACCGTTGCGCCAGCCCTGTTACTGGTCCAGGTTCAAAGAGGGATGCTCACTTCTGCGCTGTCTGCAGCGATTACGCATCGGGATATCACTATGGAGTCTGGTCGTGTGAA  360
 V  S  G  N  R  C  A  S  P  V  T  G  P  G  S  K  R  D  A  H  F  C  A  V  C  S  D  Y  A  S  G  Y  H  Y  G  V  W  S  C  E    114

GGATGTAAGGCCTTTTTTAAAAGAAGCATTCAAGGACATAATGATTATATTTGTCCAGCTACAAATCAGTGTACAATCGATAAAAACCGGCGCAAGAGCTGCCAGGCCTGCCGACTTCGG  480
 G  C  K  A  F  F  K  R  S  I  Q  G  H  N  D  Y  I  C  P  A  T  N  Q  C  T  I  D  K  N  R  R  K  S  C  Q  A  C  R  L  R    154

AAGTGTTACGAAGTGGGAATGGTGAAGTGTGGCTCCCGGAGAGAGAGATGTGGGTACCGCCTTGTGCGGAGACAGAGAAGTGCCGACGAGCAGCTGCACTGTGCCGGCAAGGCCAAGAGA  600
 K  C  Y  E  V  G  M  V  K  C  G  S  R  R  E  R  C  G  Y  R  L  V  R  R  Q  R  S  A  D  E  Q  L  H  C  A  G  K  A  K  R    194

AGTGGCGGCCACGCGCCCCGAGTGCGGGAGCTGCTGCTGGACGCCCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCGCCCCATGTGCTGATCAGCCGCCCCAGTGCG  720
 S  G  G  H  A  P  R  V  R  E  L  L  L  D  A  L  S  P  E  Q  L  V  L  T  L  L  E  A  E  P  P  H  V  L  I  S  R  P  S  A    234

CCCTTCACCGAGGCCTCCATGATGATGTCCCTGACCAAGTTGGCCGACAAGGAGTTGGTACACATGATCAGCTGGGCCAAGAAGATTCCCGGCTTTGTGGAGCTCAGCCTGTTCGACCAA  840
 P  F  T  E  A  S  M  M  M  S  L  T  K  L  A  D  K  E  L  V  H  M  I  S  W  A  K  K  I  P  G  F  V  E  L  S  L  F  D  Q    274

GTGCGGCTCTTGGAGAGCTGTTGGATGGAGGTGTTAATGATGGGGCTGATGTGGCGCTCAATTGACCACCCCGGCAAGCTCATCTTTGCTCCAGATCTTGTTCTGGACAGGGATGAGGGG  960
 V  R  L  L  E  S  C  W  M  E  V  L  M  M  G  L  M  W  R  S  I  D  H  P  G  K  L  I  F  A  P  D  L  V  L  D  R  D  E  G    314

AAATGCGTAGAAGGAATTCTGGAAATCTTTGACATGCTCCTGGCAACTACTTCAAGGTTTCGAGAGTTAAAACTCCAACACAAAGAATATCTCTGTGTCAAGGCCATGATCCTGCTCAAT  1080
 K  C  V  E  G  I  L  E  I  F  D  M  L  L  A  T  T  S  R  F  R  E  L  K  L  Q  H  K  E  Y  L  C  V  K  A  M  I  L  L  N    354

TCCAGTATGTACCCTCTGGTCACAGCGACCCAGGATGCTGACAGCAGCCGGAAGCTGGCTCACTTGCTGAACGCCGTGACCGATGCTTTGGTTTGGGTGATTGCCAAGAGCGGCATCTCC  1200
 S  S  M  Y  P  L  V  T  A  T  Q  D  A  D  S  S  R  K  L  A  H  L  L  N  A  V  T  D  A  L  V  W  V  I  A  K  S  G  I  S    394

TCCCAGCAGCAATCCATGCGCCTGGCTAACCTCCTGATGCTCCTGTCCCACGTCAGGCATGCGAGTAACAAGGGCATGGAACATCTGCTCAACATGAAGTGCAAAAATGTGGTCCCAGTG  1320
 S  Q  Q  Q  S  M  R  L  A  N  L  L  M  L  L  S  H  V  R  H  A  S  N  K  G  M  E  H  L  L  N  M  K  C  K  N  V  V  P  V    434

TATGACCTGCTGCTGGAGATGCTGAATGCCCACGTGCTTCGCGGGTGCAAGTCCTCCATCACGGGGTCCGAGTGCAGCCCGGCAGAGGACAGTAAAAGCAAAGAGGGCTCCCAGAACCCA  1440
 Y  D  L  L  L  E  M  L  N  A  H  V  L  R  G  C  K  S  S  I  T  G  S  E  C  S  P  A  E  D  S  K  S  K  E  G  S  Q  N  P    474

CAGTCTCAGTGACGCCTGGCCCTGAGGTGAACTGGCCCACAGAGGTCACAAGCTGAAGCGTGAACTCCAGTGTGTCAGGAGCCTGGGCTTCATCTTTCTGCTGTGTGGTCCCTCATTTGG  1560
 Q  S  Q  -
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B

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ERα      - MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYGPGSEAAAFGSNGLGGFPPL -100
ERβ      - MNYSIPSNV----------------------------------------------------------------TNLEGGPGRQTTSPNVLWPTPGHLS -34

ERα      - NSVSPSPLMLLHPPPQLSPFLQPHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGRERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVW -200
ERβ      - PLVVHRQLSHLYAEPQKSPWCEARSLE----------------------HTLPVNRETLKRKVSGNRCASPVTGPGSKRDAHFCAVCSDYASGYHYGVW -111

ERα      - SCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLWPSPLMIKR -300
ERβ      - SCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMVKCGSRRERCGYRLVRRQRSADEQLHCAGKAKRSGGHAPRV--------- -202

ERα      - SKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPG -400
ERβ      - --RELLLDALSPEQLVLTLLEAEPPHVLIS-RPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMMGLMWRSIDHPG -299

ERα      - KLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLMAKAGLTLQQQ -500
ERβ      - KLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILLNSSMYPLVTATQDA-DSSRKLAHLLNAVTDALVWVIAKSGISSQQQ -398

ERα      - HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGSTSSHSLQKYYITGEAEGFPATV -595
ERβ      - SMRLANLLMLLSHVRHASNKGMEHLLNMKCKNVVPVYDLLLEMLNAHVLRGCKSSITGSECSPAEDSKSKEGSQNPQSQ                -477
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Fig. 1. (A) Nucleotide sequence and deduced amino acid sequence of ERβ. (B) Alignment of the amino acid sequences of the human ERβ with the human ERα. The DNA-binding domains are shown in bold, the consensus sequence for MAPK-mediated serine phosphorylation is underlined and the F-domains are shown in italics.

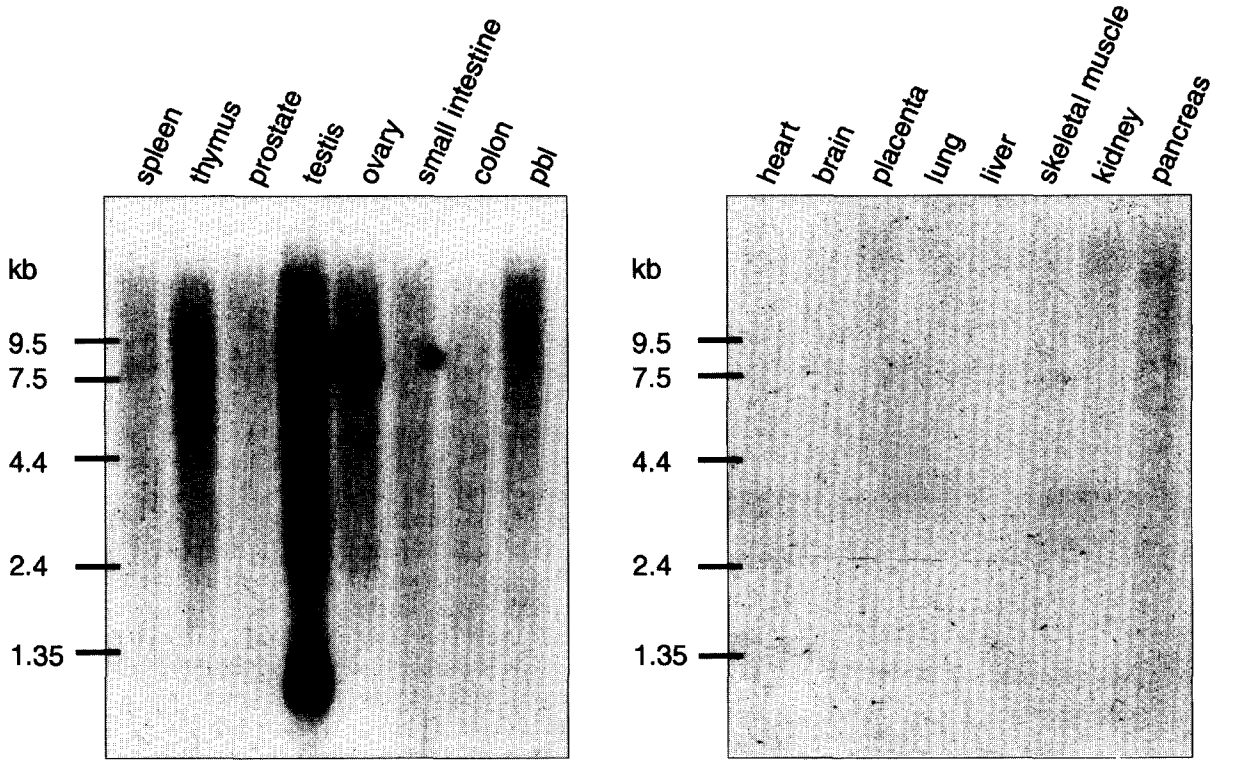

Fig. 2. Tissue distribution of ERβ. Each lane contains 2 μg of poly A+ RNA isolated from the human tissues indicated at the top (PBL, peripheral blood leukocytes). RNA size markers are indicated on the left.

Wakeling, Zeneca Pharmaceuticals, Macclesfield, UK. Cell extracts were made 48 h post-transfection by the addition of 200 μl lysis buffer (0.1 M Na_2HPO_4, 0.2% Triton X-100). 20 μl sample was added to 50 μl luciferase assay reagent (Promega). Light emission was measured in a Topcount (Packard). Beta-galactosidase activity was measured using a Galacto-light plus kit (Tropix) to correct for differences in transfection efficiency.

3. Results

Using degenerate primers (see Section 2) we cloned a PCR fragment which corresponds to a novel receptor (designated ERβ) which is highly homologous with the classical estrogen receptor (ERα). Part of this fragment (corresponding to nucleotides 484–815 in Fig. 1) was used as a probe to determine the tissue distribution of ERβ by Northern blot analysis. Fig. 2 shows that ERβ is expressed in thymus, testis and ovary (approx. 8 kb and 10 kb transcripts) and very faintly also in spleen. In addition, a relatively abundant transcript of 1.3 kb is present in testis. Screening of a human testis cDNA library resulted in the identification of two identical positive clones which contained a large part of the ERβ reading frame (nucleotides 1–1265 in Fig. 1A). At the 5′ end, an in-frame ATG codon (nucleotides 19–21) is present, but since the clone lacks an in-frame stop codon further upstream, we cannot exclude the possibility that upstream ATG translation initiation codons may be present. At the 3′ end, the homology with the ERα is lost abruptly at a site which corresponds to the splice site between exon 7 and exon 8 in ERα, suggesting that this cDNA is derived from an incompletely spliced transcript. In order to obtain nucleotide sequences downstream of this splice site, testis cDNA (Marathon-ready, Clontech) was used as a template to generate a RACE PCR fragment that was cloned and sequenced. It was shown to contain the carboxy-terminus of ERβ, including AF-2, an F-domain followed by a stop codon and part of the 3′ untranslated region. A comparison of the complete ERβ and ERα amino acid sequences is shown in Fig. 1B.

The high degree of conservation of the DNA-binding domain (96%) and the relatively high degree of conservation of the ligand-binding domain (58%), as compared to currently reported estrogen receptor-related receptors ERR1 and ERR2 [13], suggested that ERβ could bind estrogenic hormones and activate transcription of ERE-containing promoters. To experimentally test this hypothesis, CHO cells were transfected with an ERβ (or ERα) expression construct together with a well-known ERα-responsive reporter plasmid (rat oxytocin promoter-luciferase, RO-luc [14]) and treated with 17β-estradiol. Fig. 3A clearly shows that ERβ can activate transcription of RO-luc in a hormone-dependent manner, although the level of induction is higher for ERα. In addition, ERβ is able to activate transcription of a reporter construct containing three synthetic EREs (not shown). Fig. 3A also shows that the ERα antagonist ICI-164384 (ICI) is a potent antagonists for both ERα and ERβ. Fig. 3B shows a dose-dependent transactivation of ERα and ERβ by 17β-estradiol, indicating that both receptors can be activated at very low hormone concentrations. However, half-maximal activation is reached at lower concentrations of 17β-estradiol for ERα than for ERβ.

4. Discussion

The current report describes the identification of ERβ as a functional estrogen receptor which can interact with the re-

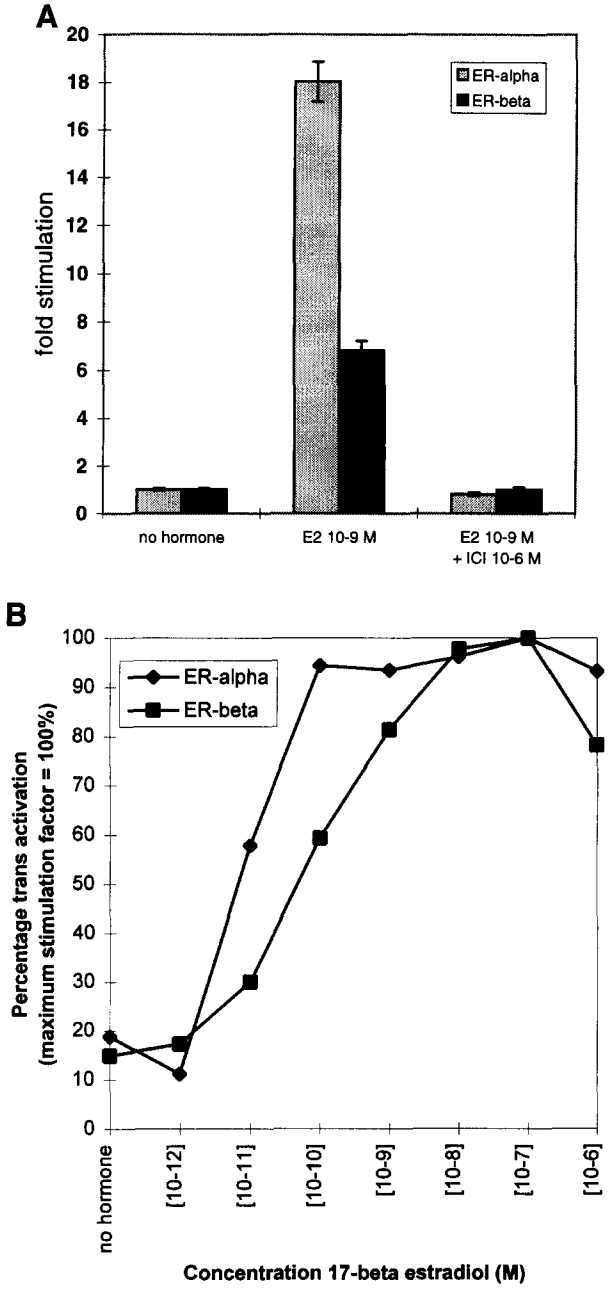

Fig. 3. (A) Effect of 17β-estradiol (E2) alone or in combination with the anti-estrogen ICI-164384 (ICI) on the transcriptional activity of ERα (gray bars) and ERβ (black bars) using an ERE-based reporter gene. Indicated is the fold induction of the luciferase activity of cells receiving no hormone. All luciferase activities were determined in triplicate and were normalized for differences in transfection efficiency by measuring β-galactosidase activity in the same lysate. (B) Concentration-dependent transactivation of ERα and ERβ by 17β-estradiol. Transactivation at 10^{-7} M was arbitrarily set at 100%.

ference ERα agonist (17β-estradiol) as well as a widely used antagonist (ICI-164384). ERβ was demonstrated to activate transcription of ERE-containing promoters. Consequently, a number of experimental in vitro and in vivo data, including those concerning the ERα knock-out mice [15], will have to be re-evaluated.

A comparison of ERβ with ERα reveals a number of interesting aspects. The tissue distribution of ERβ appears to be restricted to thymus, spleen, ovary and testis. However, the initial PCR fragment was cloned from peripheral blood lymphocytes, whereas no signal is detected on Northern blot. Therefore, it may be that the sensitivity of Northern blot analysis is insufficient to detect ERβ expression in some tissues. It is not likely that the probe and the conditions used allow hybridization with ERα. Hybridization of the same blots with a probe corresponding to part of the 3′ untranslated region of ERα revealed a 6.5 kb band in almost all tissues, especially ovary, testis, prostate and skeletal muscle (not shown). The ligand-binding domain is relatively well conserved (58%) and our data demonstrate that as a result, ERβ closely resembles ERα with respect to the binding of 17β-estradiol and ICI-164384. This suggests that there is a high degree of structural conservation of the ligand-binding pocket(s). Although both receptors interact with 17β-estradiol with high affinity, Fig. 3B suggests that ERα has a higher affinity for this ligand than ERβ. The difference with respect to the level of induction may relate to the nature of the ligand, the responsive element or to the cellular context (co-activators/repressors), which may not have been optimal for ERβ in the current co-transfection assay. Although the DNA-binding domain is extremely well conserved (96%), ERβ and ERα may activate different sets of target genes. Since not only the P-box but also the D-box is conserved, it can be envisaged that in addition to forming homodimers, ERβ and ERα may form heterodimers thereby influencing each other's transcriptional activity.

The A/B domain, the hinge (domain D) and the F-domain are poorly conserved. Interestingly however, the ERβ A/B domain contains a consensus sequence for MAPK-mediated serine-phosphorylation (PQKSP in ERβ, underlined in Fig. 1B) which is also present and functional in the ERα A/B domain [16]. The lack of conservation of the A/B domain suggests important differences between both receptors with respect to their AF-1 activity and with respect to interactions of co-activators and/or repressors with this region. The lack of conservation of the F-domain is also striking in view of its suggested role in modulating the extent of the agonistic activity of compounds like 4-OH-tamoxifen in ERα [17].

In conclusion, the cloning of ERβ adds to the complexity of (tissue-specific) estrogenic effects. It can be envisaged that it will be worthwhile to dissect ERβ pathways from ERα pathways as well as to identify compounds which specifically interact with ERα or ERβ.

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