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Estrogen Receptors Beta4 and Beta5 Are Full Length Functionally Distinct ERβ Isoforms

Cloning from Human Ovary and Functional Characterization

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We describe here the cloning and functional characterization of two unique ER isoforms, ERβ4 and ERβ5. The full length ERβ4 and ERβ5 were identified by asymmetric PCR using human ovary cDNA, cloning, and sequence analyses. Both receptors share identical sequences with ERβ1 from exon 1 to exon 7. In the place of exon 8, ERβ4 has unique sequences arising from a region downstream of the ERβ gene and upstream of the SYNE2 gene. ERβ5 has sequences arising from retention of the 5' end of the intron between exon 7 and 8. Both receptors bind promoter sequences on DNA but do not bind estrogen. They translocate to the nucleus and exhibit three to four times higher estrogen-independent transcriptional activity than ERβ1. When co-transfected with ERα, they predominantly form heterodimers and negatively regulate its transcriptional activity. Estrogen-independent transcriptional activity of ERβ5, but not ERβ4, was inhibited by ERα, demonstrating for the first time that ERα regulates ERβ. Tissue-specific expression of ERβ4 and ERβ5, together with their ligand-independent transcriptional properties and ERα modulating activities, could have a number of implications in seemingly unlinked biological processes regulated by estrogen.

Key Words: Asymmetric PCR; estrogen receptor beta; estrogen receptor alpha; ligand-independent transcription; ERE-binding; negative modulators of ER; full-length ERβ4 and ERβ5.

Introduction

The steroid hormone estrogen mediates a wide variety of complex biological processes in a diverse range of tissues from reproductive tracts of both males and females and mammary tissues to non-reproductive tissues from cardiovascular, skeletal, immune, nervous, gastrointestinal, and urinary systems *(1)*. In addition to maintenance of normal functions of the above systems, estrogens are implicated as causative agents for developing breast cancers *(2)* and the loss of estrogen after menopause for osteoporosis in women. Hence, there is an immense interest in understanding the molecular mechanisms of actions of this hormone in a broad range of seemingly unlinked biological processes.

Recent progress in the understanding of the molecular mechanisms of estrogen signal transduction has revealed that different cells respond to the same hormone differently because of the differences in the expression levels of a number of proteins that directly or indirectly interact with ERα, the high-affinity receptor for estrogen *(3)*. The estrogen-bound ERα is a transcription factor, which upon binding to target gene promoters activates the transcription of genes under estrogen control. It is expressed in all target tissues and is required for estrogen action. Direct interaction of ERα with co-activator or co-repressor molecules results in either positive or negative regulation of gene transcription. One important molecule that directly interacts with ERα and negatively regulates estrogen signaling is the structurally similar but genetically distinct ERβ. It is encoded by a different gene *(4,5)* and shares homologous sequences in the DNA- and hormone-binding regions (96% and 60% respectively) with ERα *(5)*. ERβ interacts with identical promoters on DNA, exhibits similar binding affinity profiles with a number of natural and synthetic ligands *(6)*, and activates gene transcription when bound to estrogen. Although ERα and ERβ were originally thought to have redundant functions, a number of distinct structural and functional properties demonstrated that ERβ is a negative regulator of estrogen action (reviewed in ref. *7*). ERβ lacks most of the N-terminal AF-1 (activation function 1) domain. The C-terminal AF-2 domain that interacts with co-activators and co-repressors functions differently from the similar ERα domain and does not activate gene transcription at the AP-1 promoter site when bound with ERα agonists *(8)*. When ERβ is co-transfected with ERα, it forms heterodimers with ERα on DNA and acts as an efficient dominant inhibitor of ERα activity *(9)*. The in vitro observations were supported by in vivo results that sensitivity to estradiol was increased in the uteri of ERβ knockout mice *(10)*.

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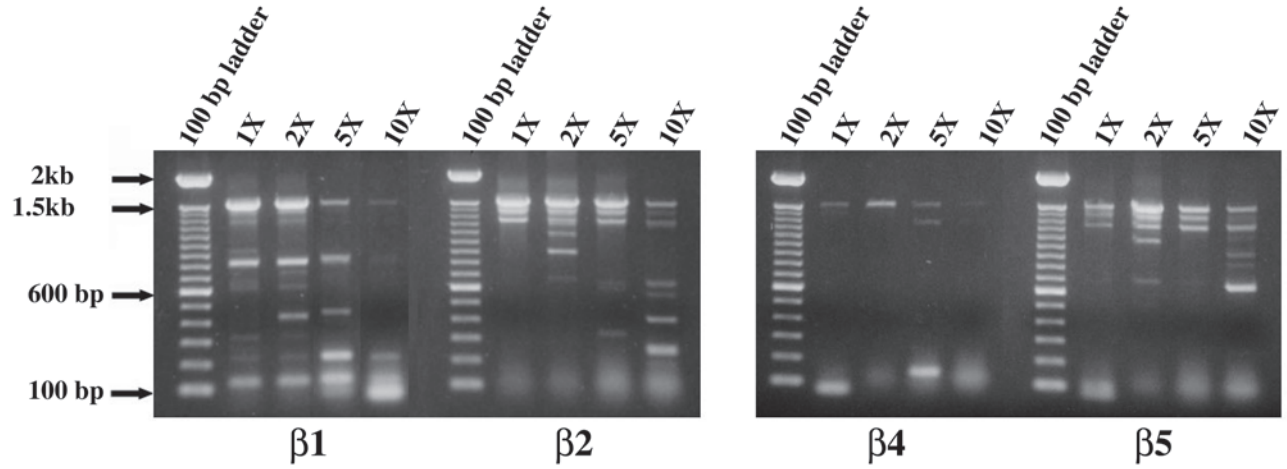

Fig. 1. Amplification of full-length ERβ4 and ERβ5 coding sequences by asymmetric PCR. The full-length ERβ4 and ERβ5 coding sequences were amplified using human ovary cDNA, a sense primer upstream of the translation initiation site of ERβ1 and specific anti-sense primers downstream of stop codons for ERβ4 and ERβ5 as described in methods section. The optimum amplification conditions were determined by conducting PCR in the presence of varying concentrations of sense primer (1–10 μ*M*, 1X–10X) and a constant concentration (1 μ*M*, 1X) of anti-sense primers. For comparative purposes, ERβ1 and ERβ2 sequences were also amplified using isoform specific anti-sense primers. The PCR products (12.5 μL) were separated by electrophoresis in 1% Nu Sieve agarose gels and stained with ethidium bromide. PCR products amplified for four ERβ isoforms are shown. Amplifications of full-length ERβ4 and ERβ5 (1662 and 1517 bp, respectively) were most efficient when the sense primer concentration was 2X. For ERβ1 and ERβ2 amplifications (1527 and 1517 bp, respectively), the optimum sense primer concentration was 1X or 2X. In addition to the full-length products, several smaller products, presumably exon deletion variants, were also amplified.

Tissue expression patterns of ERα and ERβ also suggested that ERβ plays a regulatory role in the degree of response to estrogen in different tissues. ERβ predominates in tissues that do not have heavy estrogen dependence such as the male reproductive organs and nonreproductive tissues. ERα, on the other hand, predominates in the female reproductive tracts, the major target tissues of estrogen action. However, in ovary, the primary site of estrogen production, the level of ERβ equals that of ERα *(11)*.

The tissue-specific regulation of ERα by ERβ appears to be complex in that it is not through one species of ERβ, but through several closely related functionally distinct isoforms. In addition to wild-type ERβ1, an isoform, ERβ2, was reported to negatively regulate estrogen signaling by forming heterodimers with ERα and inhibiting its activity. ERβ2 does not bind estrogen or DNA and is co-expressed with ERβ1 in most of the estrogen target tissues *(12–14)*. Two other isoforms, ERβ4 and ERβ5 were also described *(12)*. However, these two receptors were considered to have no role in estrogen signaling because they were detected as short sequences spanning from the 3' end of exon 7 and short unique sequences in the place of exon 8 *(12)*. Our recent study on profiling of various isoforms of ERβ in ovary, breast, bone and uterus tissues *(15)* demonstrated that ERβ5 was expressed at higher levels than ERβ1 and ERβ4 expression was specific to ovary. Our preliminary results also suggested that both ERβ4 and ERβ5 might be full length molecules similar to ERβ1 and ERβ2. These observations led us to investigate the structures of ERβ4 and ERβ5 and their role in estrogen signaling. We demonstrate here for the first time that ERβ4 and ERβ5 are full length molecules with unique functional properties distinct from ERβ1 or ERβ2. We also established that both receptors have significant estrogen independent transcriptional properties and are strong negative modulators of ERα.

Results

The ERβ4 and ERβ5 are Full-Length Receptors with Unique Exon 8 Sequence

We applied asymmetric PCR to investigate whether ERβ4 and ERβ5 are full-length receptors, similar to ERβ1, ERβ2, and ERβ3. We used a sense primer upstream of the translation start site of ERβ1 *(17)*, and isoform-specific anti-sense primers downstream of translation stop codons based on previously reported sequences *(12)*. Because the sense primer could anneal to all four (ERβ1, ERβ2, ERβ4, and ERβ5) transcripts, it will be a limiting factor in a PCR for optimal amplification of any one of the receptor cDNAs. Therefore, we tested a novel amplification procedure, namely, asymmetric PCR, in which amplifications were conducted in the presence of constant concentrations of anti-sense primers that were specific to ERβ4 and ERβ5 and increasing concentrations of sense primer using human ovary cDNA. For comparative purposes we also conducted asymmetric PCR with ERβ1 and ERβ2 specific anti-sense primers. The results obtained using four anti-sense primers specific for ERβ1, ERβ2, ERβ4, and ERβ5 in the presence of increasing amounts of sense primer are shown in Fig. 1. The ERβ1 anti-sense primer generated equal amounts of a major 1527 bp PCR prod-

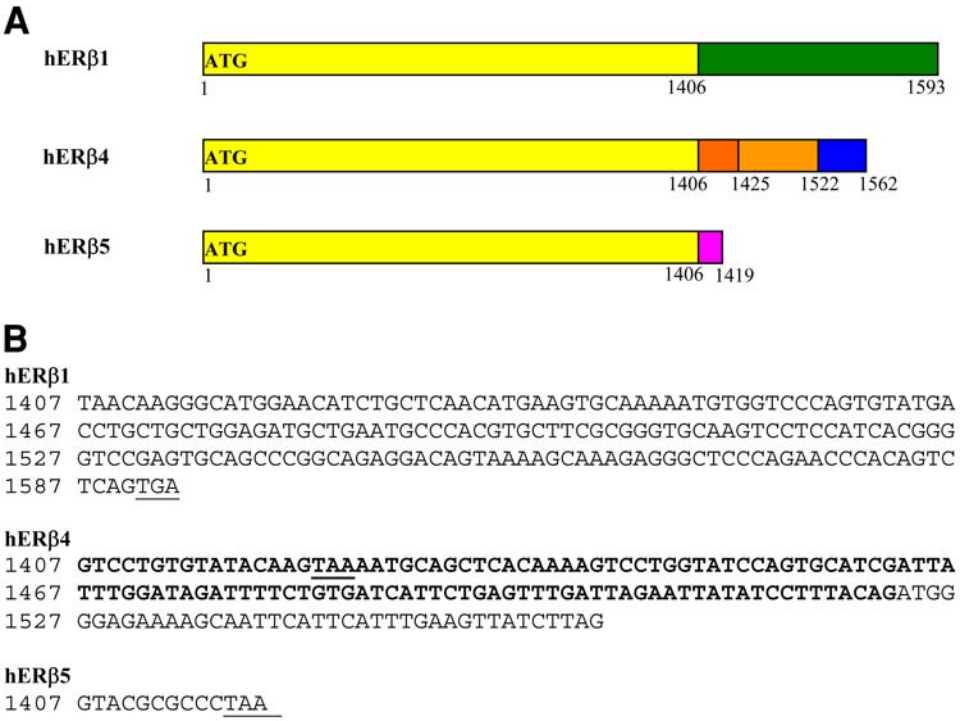

Fig. 2. Full-length nucleotide sequences of ERβ4 and ERβ5. To derive the identity of the 1662 and 1517 bp PCR products amplified using ERβ4 and ERβ5 specific anti-sense primers together with a sense primer upstream of translation initiation site of ERβ1, they were gel purified, cloned into pCR®3.1 vector, and sequenced using a number of sense and anti-sense primers as described in methods section. Full-length sequences were deduced from a number of overlapping nucleotide sequences and compared with ERβ1. (**A**) Schematic representation of homologous sequences in ERβ1, ERβ4, and ERβ5. Both ERβ4 and ERβ5 showed sequences identical to ERβ1 from the translation initiation start codon, ATG, to the end of exon 7 (bp 1406), and diverged thereafter. ERβ1 exon 8 spans from bp 1407 to 1593. ERβ4 has an insert of 116 bp (1407–1522), which was not previously reported. The coding sequence in this insert was 16 bp (1407–1425). The 116 bp insert was followed by the sequence reported by Moore et al. *(12)*. The ERβ5 exon 8 coding sequence is from bp 1407 to 1419 and was identical to previously reported. (**B**) Specific coding nucleotide sequences beyond exon 7 in ERβ4 and ERβ5 in comparison with ERβ1 are shown. Translation stop codons are underlined. The previously unreported sequence (116 bp) is shown in bold.

uct with 1X (1 μ*M*) and 2X concentrations of the sense primer, but the products decreased rapidly when the concentrations were increased to 5X and 10X. The ERβ2-specific anti-sense primer generated equal amounts of 1517 bp product with 1X, 2X, and 5X concentration and the product was diminished with 10X concentration. Upon sequence analysis, the 1527 and 1517 bp products generated with ERβ1 and ERβ2 specific anti-sense primer, respectively, were found to be coding sequences of the above receptors as reported in the literature *(12,13,17)*.

The ERβ4 specific anti-sense primer along with 1X concentration of sense primer generated a small but detectable 1662 bp product, which increased when the primer concentration was increased to 2X, but decreased with 5X and 10X. ERβ5-specific anti-sense primer generated 1517 bp product in a similar pattern as ERβ4, except the amounts were far higher than for ERβ4. In addition to the above major products, all the primer pairs generated several lower-molecular-weight products, presumably the exon deletion splice variants. Interestingly, the ERβ2- and ERβ5-specific primer pairs generated a greater number of splice variant products than the other two primer pairs (Fig. 1).

The 1662 and 1517 products generated by ERβ4- and ERβ5-specific anti-sense primers, respectively, were gel purified and cloned into pCR®2.1-TOPO. For the purpose of expression, the cloned plasmids were re-amplified using a sense primer containing Kozak sequence upstream of the translation initiation codon, and the products cloned into an expression vector, pCR®3.1. The cloned pCR®3.1 plasmids containing ERβ4 and ERβ5 were sequenced using a number of primers listed in the Methods section. Sequence analyses generated overlapping sequences. The deduced full-length sequences are presented schematically in comparison with ERβ1 coding sequence in Fig. 2A and the actual exon 8 sequences in Fig. 2B. As shown in the figure, the ERβ4 and ERβ5 are full-length sequences identical with ERβ1 from translation start codon through the end of exon 7. Beyond exon 7 they diverged as shown previously by Moore et al. *(12)*. However, ERβ4 had an additional 116 bp insert upstream of the sequence reported previously *(12)*. ERβ5 sequence after exon 7 was identical to the sequence as reported *(12)*. These results demonstrate for the first time that ERβ4 and ERβ5 are full-length receptors contrary to the previous reports.

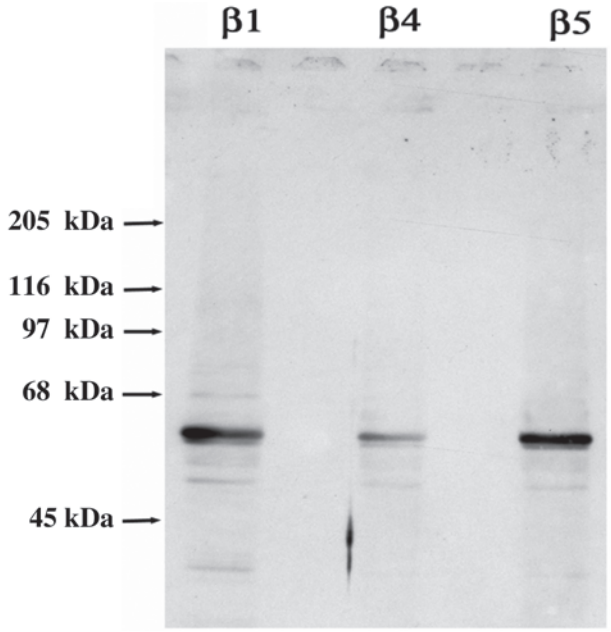

Fig. 3. Western blotting of ERβ4 and ERβ5 recombinant proteins expressed in COS-7 cells with antibodies against ERβ. The whole-cell extracts from COS-7 cells that were transiently transfected with full-length ERβ4 and ERβ5 cDNA plasmids. The cell lysates were separated by SDS-PAGE overnight at a constant current of 20 mA. The proteins were transblotted to nitrocellulose filters, probed with polyclonal antibodies against ERβ1 (H-150), and the antigen–antibody complexes were detected using ECL reagent. For comparative purposes, whole-cell extracts from cells transfected with ERβ1 cDNA plasmids were also Western blotted as above. ERβ4 and ERβ5 proteins migrated slightly faster than ERβ1 and all were recognized by the polyclonal antibodies as expected.

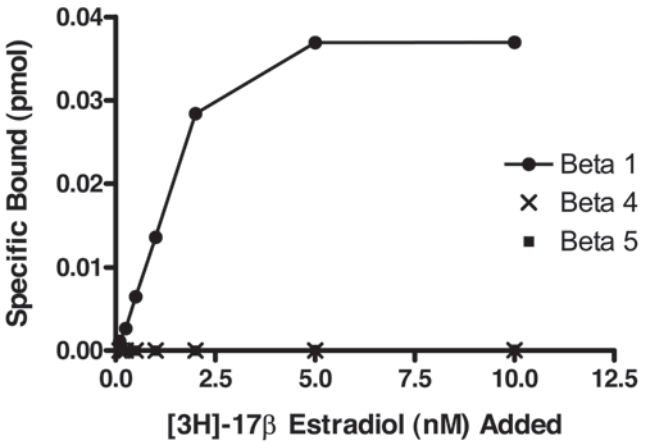

Fig. 4. Ligand-binding properties of ERβ4 and ERβ5 proteins. Estrogen-binding properties of ERβ4 and ERβ5 were tested using whole-cell extracts of COS-7 cells transfected with their respective cDNA plasmids and ^{3}H-labeled estrogen as described in methods. The COS-7 cell extracts prepared by transfecting ERβ1 plasmid showed specific binding, but extracts from ERβ4 and ERβ5 transfected cells did not show any binding to the labeled ligand.

A Blast search of human genome sequence showed that the specific coding nucleotide sequence of ERβ5 arose by the retention of the beginning of intron between exon 7 and exon 8 (chromosome 14, bp, 44621669–44621728). The ERβ4 specific sequence was found in a non-coding region downsteam of the ERβ gene and upstream of the spectrin repeat containing nuclear envelope gene, SYNE2 (chromosome 14, bp 44614601–44614804). We also searched for ERβ2- and ERβ3-specific coding sequences. We found that ERβ2 exon 8 sequences were from a non-coding region downsteam of the ERβ gene and upstream of the SYNE2 gene (chromosome 14, bp 44614173–44614374). The ERβ3 exon 8 sequences were found in intron 15 of the SYNE2 gene (chromosome 14, bp 44471679–44471811).

The plasmids with full-length ERβ4 and ERβ5 coding sequences were transiently expressed in COS-7 cells, the expressed proteins were separated by SDS-PAGE, transblotted to nitrocellulose filters, and probed with rabbit polyclonal antibodies against the N-terminal portion of ERβ (H-150). The results are presented in Fig. 3. In comparison to the ERβ1 protein that was expressed with a pCXN2 plasmid, the ERβ4 and ERβ5 proteins showed slightly higher mobility on SDS-PAGE as expected from their smaller size. The apparent molecular weights obtained for both were 55 kDa as would be expected for the 474- and 472-amino-acid peptides predicted from their coding sequence. These results further support our conclusion that ERβ4 and ERβ5 are full-length receptors. During SDS-PAGE analyses of recombinant ERβ4 and ERβ5 as well as ERβ1 proteins, we observed anomalous migration patterns of these proteins. They often migrated as several bands of inconsistent molecular weights presumably due to incomplete reduction of intra-disulfide bonds formed by a large number of half cysteines (data not shown).

ERβ4 and ERβ5 Specifically Bind Promoter Sequences on DNA but Do Not Bind Estrogen

Because both receptors retain most of the ligand binding domain, we tested if they bind estrogen. Binding to estrogen was performed with [^{3}H]17β estradiol and whole-cell extracts prepared from ERβ4 and ERβ5 transfected COS-7 cells. Extracts prepared by transfection of ERβ1 cDNA were used as a positive control. We found that neither ERβ4 nor ERβ5 showed measurable binding with radiolabeled estradiol. In similar experiments conducted with extracts prepared from ERβ1 transfected cells, specific binding with a K_d value of 5×10^{-10} M was observed (not shown). The specific binding versus the amount of the ligand added is shown in Fig. 4. These results showed that truncation of exon 8 and retention of a portion of intron 7 (ERβ5) or substitution with unrelated sequences (ERβ4) totally abolished their estrogen-binding properties. After establishing that both receptors do not bind estrogen, we next tested whether they bind to promoter sequences using radiolabeled ERE sequences and a gel shift mobility assay. We used ERβ1 transfected cell extracts and MCF-7 cell extracts as positive controls. Inter-

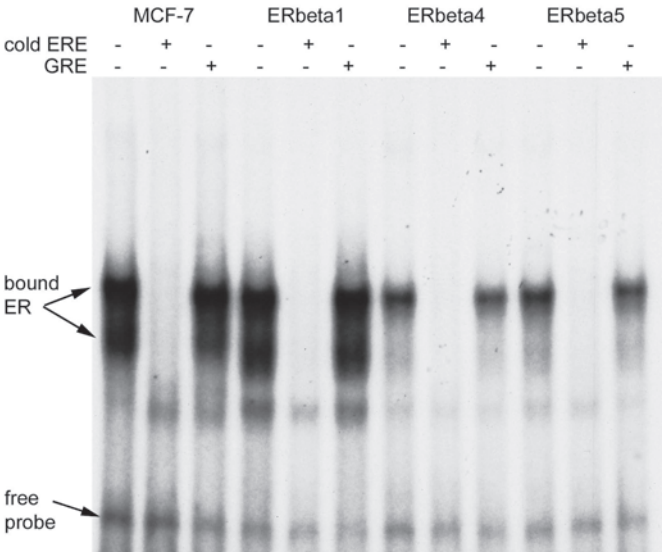

Fig. 5. Recombinant ERβ4 and ERβ5 proteins bind promoter sequences on DNA. The recombinant proteins expressed in COS-7 cells were tested using the whole cell extracts and ^{32}P-labeled ERE oligonucleotide by gel mobility shift assay as described in the Methods section. The extracts were incubated with ^{32}P-labeled ERE oligonucleotide and the bound label was separated from the free by electrophoresis on 5% pre-run polyacrylamide gels. MCF-7 cell extracts and COS-7 cells transfected with ERβ1 plasmid were used as positive controls. Specific binding was determined by competition with excess amounts (3 p*M*) of cold ERE and non-specific binding with cold GRE. The recombinant ERβ4 and ERβ5 protein binding to ^{32}P-labeled ERE was similar to that of MCF-7 and ERβ1 transfected cell extracts. This binding was specifically inhibited by cold ERE but not cold GRE.

estingly, both ERβ4 and ERβ5 transfected whole-cell extracts showed binding to [^{32}P]-labeled ERE (Fig. 5) similar to ERβ1 and ERα (MCF-7 cell extract). This binding was totally abolished when conducted in the presence of cold ERE, but not with cold glucocorticoid response elements (GRE). These results demonstrated that ERβ4 and ERβ5 possess specific promoter binding properties.

Both ERβ4 and ERβ5 Translocate to the Nucleus

To investigate whether the cloned full-length recombinant ERβ4 and ERβ5 proteins translocate to the nucleus, COS-7 cells that were transiently transfected with their respective cloned plasmids were fixed and probed with two different antibodies directed against the N-terminal portion of ERβ. The immunostaining pattern with polyclonal (H-150) (left panel) and monoclonal (β14C8) (right panel) antibodies are presented in Fig. 6. For comparative purposes, immunolocalization of ERβ1 is also shown. The polyclonal antibodies stained nuclear membranes and cytosols of COS-7 cells that were transfected with ERβ1 (Fig. 6, top left panel). The monoclonal antibodies stained nuclei of ERβ1 transfected cells (Fig. 6, top right panel). Both antibodies stained the nuclei and cytosols of COS-7 cells transfected with ERβ4 and ERβ5 plasmids (second and third panels, respectively). These results demonstrated that both ERβ4 and ERβ5 proteins translocate to the nucleus, therefore, could activate gene transcription.

ERβ4 and ERβ5 Activate Gene Transcription on an ERE Independent of Estrogen

To investigate whether the above two receptors mediate gene transcription on ERE promoter sites, we used a CAT reporter gene containing two copies of an ERE and the viral thymidine kinase promoter driving the expression of the CAT reporter gene for transactivation studies. We performed co-transfection experiments in which COS-7 cells were co-transfected with expression plasmids for ERβ1 or ERβ2 or ERβ4 or ERβ5 and the above estrogen responsive reporter gene construct. In the absence of any estrogen, ERβ1, ERβ4, and ERβ5 showed transciptional activity although at different levels. ERβ4 and ERβ5 showed about three times higher activity than ERβ1 in the absence of estrogen. Upon exposure to 100 n*M* 17β estradiol (E_2), the transcriptional activity of ERβ1 increased about 10-fold, whereas the activities of ERβ4 and ERβ5 did not change significantly. We did not observe any increase in the activities of ERβ4 and ERβ5 even when the concentration of estrogen was increased to 1000 n*M* (data not shown). Unlike the other three receptors, ERβ2 protein did not exhibit any activity either in the absence or after the addition of E_2 up to 1000 n*M*. The relative CAT activities of ERβ1, ERβ2, ERβ4, and ERβ5 are presented in Fig. 7.

ERβ4 and ERβ5 form Heterodimers with ERα

Because both ERβ4 and ERβ5 retain dimerization domains, we tested whether they heterodimerize with ERα by gel mobility shift assay. For testing this, we performed co-transfection experiments in which COS-7 cells were transfected with either an expression vector for ERβ4 or ERβ5 and the plasmid with cloned ERα cDNA. The expression of ERα and ERβ4, or ERα and ERβ5, in double-transfected cell extracts was confirmed by Western blotting (see Fig. 9). The receptors in the whole-cell extracts were allowed to interact with radiolabeled ERE and analyzed by PAGE. The gel mobility shifts with extracts from double-transfections were compared with single-transfection products (Fig. 8). The ERβ4 and ERα homodimers showed different mobilities. However, the extracts from ERβ4 and ERα double-transfected cells moved as a single species, demonstrating heterodimer formation between these two receptors (Fig. 8, panel A). In the case of ERβ5, the extract from double-transfected cells migrated as a fast moving single species distinct from either of the homodimers (Fig. 8, panel B). These results demonstrate that both ERβ4 and ERβ5 preferentially form heterodimers with ERα.

Both ERβ4 and ERβ5 Modulate ERα Transcriptional Activity

We next tested whether dimerization of ERβ4 and ERβ5 with ERα alters ERα transcriptional activity. For this, we

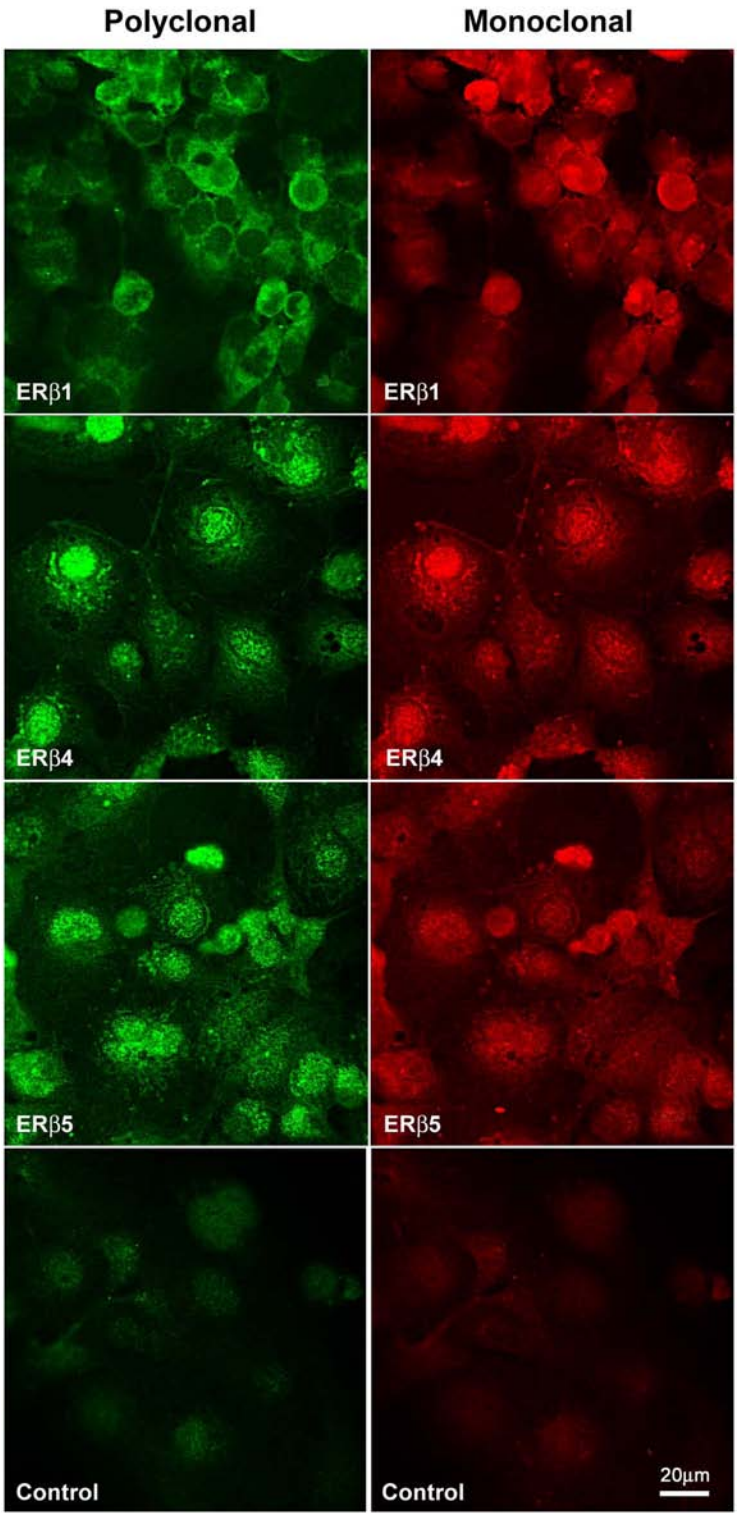

Fig. 6. ERβ4 and ERβ5 proteins were predominantly localized in nucleus. Intracellular localization of these two receptors was probed using both polyclonal and monoclonal antibodies against N-terminal portion of ERβ. COS-7 cells were transiently transfected with plasmids containing full-length ERβ4 and ERβ5 and fixed in cold acetone:methanol and were first probed with 1:100 dilution of polyclonal antibodies (H-150) and then a 1:100 diluted goat anti-rabbit IgG (H + L) conjugated to Alexa Fluor 488. This was followed by probing with 1:100 diluted monoclonal antibodies (β14C8) and 1:100 dilution of goat anti-mouse IgG (Fc Specific, TRITC conjugate Sigma T-6653) secondary antibodies. The labeled cells were visualized under confocal microscope. The left panel shows staining with polyclonal antibodies and green fluorescent secondary antibody. The right panel shows the staining with monoclonal antibody and red fluorescent secondary antibodies. Both monoclonal and polyclonal antibodies were localized in the nucleus establishing that the recombinant proteins translocate to nucleus. The bottom panel shows double-label background immunofluorescence in ERβ1 transfected cells without primary antibodies.

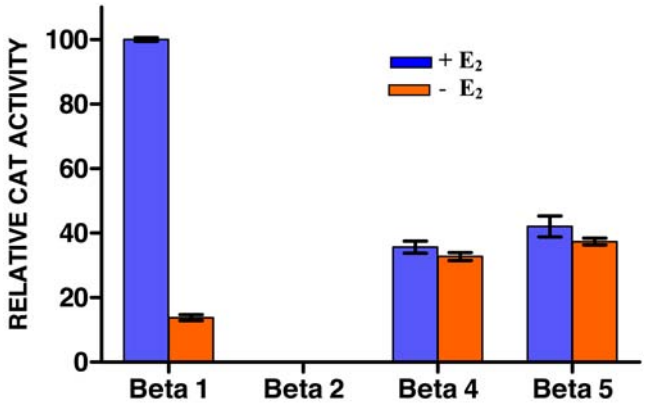

Fig. 7. Both ERβ4 and ERβ5 activate gene transcription in the absence of estrogen. To investigate whether the above two receptors mediate gene transcription on ERE promoter sites, we used a CAT reporter gene for transactivation studies. The whole-cell extracts prepared from co-transfection with expression plasmids for ERβ1 or ERβ2 or ERβ4 or ERβ5 and the CAT reporter gene were assayed for CAT as described in methods. In the absence of any estrogen, ERβ1, ERβ4, and ERβ5 showed transcriptional activity although at different levels. Upon exposure to 100 n*M* estrogen, the transcriptional activity of ERβ1 increased about 10-fold, whereas the activities of ERβ4 and ERβ5 did not change significantly. Unlike these three receptors, ERβ2 did not exhibit any activity either in the absence or after the addition of estrogen up to 1000 n*M*. The relative CAT activities of ERβ1, ERβ2, ERβ4, and ERβ5 are shown.

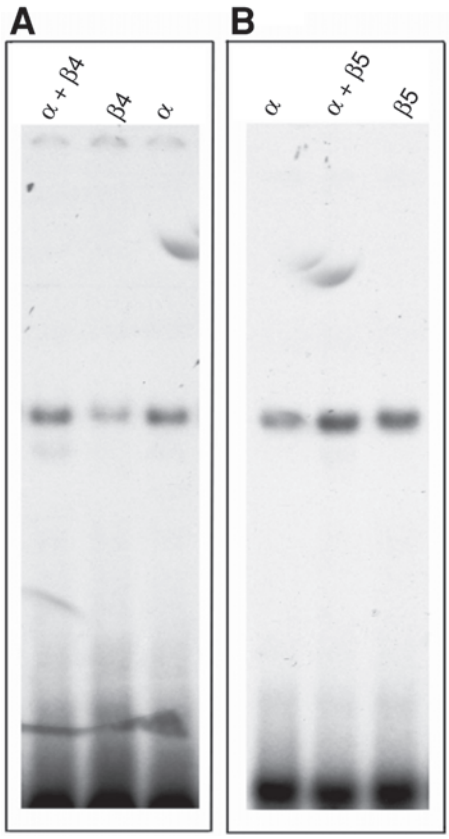

Fig. 8. ERβ4 and ERβ5 form heterodimers with ERα. For testing this property, we performed co-transfection experiments in which COS-7 cells were transfected with either an expression vector for ERβ4 or ERβ5 and the plasmid with ERα coding sequences. The presence of both proteins in the whole-cell extracts were first confirmed by Western blotting. The receptors were allowed to interact with radiolabeled ERE and analyzed by PAGE. The extracts from double-transfected cells were compared with single ER transfected extracts. The ERβ4 and ERα homodimers showed different mobilities (panel **A**) but extract from ERβ4 and ERα co-transfected cells moved as a single species, demonstrating heterodimer formation. The ERβ5 and ERα double-transfected cell extract showed a single species with higher mobility than either of the homodimers, again demonstrating heterodimer formation (panel **B**).

co-transfected estrogen-depleted COS-7 cells with plasmids containing CAT reporter gene and ERα along with ERβ4 or ERβ5 plasmids. We tested the transcriptional activity in the extracts containing varying ratios of ERα to ERβ. To obtain different molar ratios of ERβ4 or ERβ5 to ERα, the co-transfections were carried out with constant amount of ERα plasmid and varying amounts of ERβ4 or ERβ5 plasmids. After 6 h of transfection, the cells were stimulated with either E_2 or vehicle for 48 h. We chose to test the modulation in the presence of 1 n*M* E_2 because the ERα activity at this concentration was comparable to estrogen-independent activity of ERβ4 and ERβ5 (data not shown). We expected that any modulation of ERα would readily be apparent at this concentration. After stimulation with E_2, the cells were harvested, lysed, and the expression levels of ERβ4 or ERβ5 and ERα proteins in the lysates were determined by Western blotting and scanning of the blots using MCF-7 cell extract as a standard. The protein levels in the single and double ER plasmid transfected cells were determined as described earlier. The expression levels of ERβ4 or ERβ5 and ERα proteins in lysates prepared from transfections at ERα:ERβ plasmid ratios of 1:1, 1:5, and 1:10 are shown in Fig. 9. Western analyses confirmed that all three proteins were expressed in both the presence and absence of E_2 although at different levels. Unexpectedly, the ERα:ERβ protein ratios and total amounts of ER proteins did not correlate with the plasmid ratios used for transfections, presumably due to variations in transfection efficiencies. However, we observed that with increasing ERβ4 or ERβ5 plasmid concentration used for transfection, the levels of ERα protein expression was decreased.

We next determined the transcriptional activity in the lysates by assaying CAT for a given amount of protein in the lysates of single- and double-ER-transfected cells as described above. We observed that in the absence of any estrogen, ERα had no activity in agreement with previous reports. The observed CAT in all double-transfected lysates was compared with the total expected based on the individual receptor activities and their protein levels. The results obtained in the presence and absence of 1n*M* E_2 are presented in Fig. 10. In the absence of any E_2, there was no significant difference in the observed and expected activities of ERβ4 (Fig. 10, top left panel). Although it is ERα present

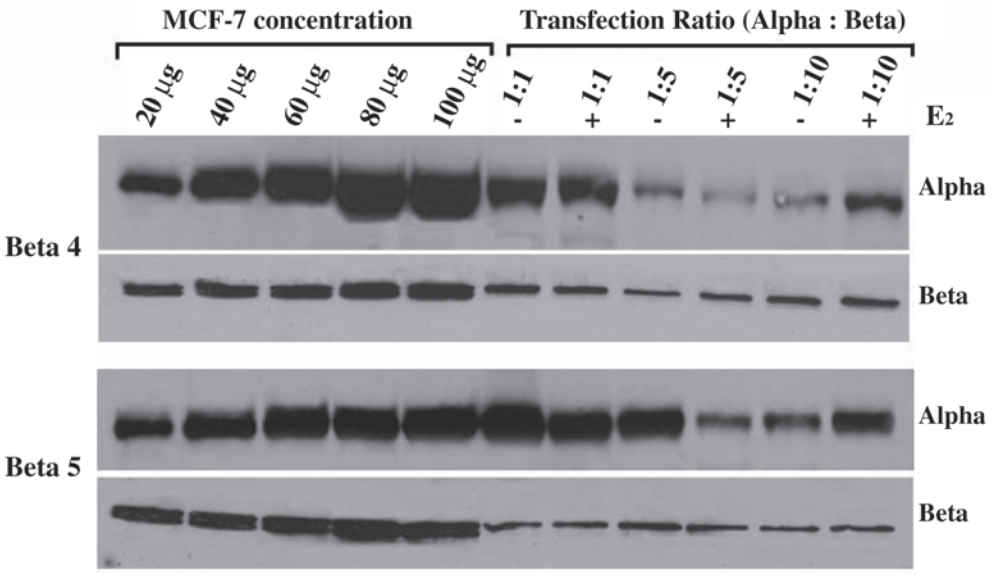

Fig. 9. Western blotting of lysates prepared from COS-7 cells transfected with ERβ4 or ERβ5, ERα and p2ERE-TK- CAT plasmids. Transfected cells (8 µL) were lysed with 300 µL of lysis buffer and centrifuged. The lysate (40 µL for ERα and 20 µL for ERβ4 or ERβ5) was electrophoresed in SDS polyacrylamide gels according to Laemmli, transblotted to nitrocellulose filters, and probed with antibodies against ERβ and ERα (H-150 and HC-20, respectively). The antigen–antibody complexes were detected using ECL. Expression levels of proteins in the lysates prepared from cells transfected with various ratios of ERα:ERβ4 or ERβ5 plasmids, and in the presence and absence of estrogen are shown. The upper two panels show Western blotting of lysates prepared from cells transfected with ERα and ERβ4 and the lower two panels show Western blotting of lysates prepared from cells transfected with ERα and ERβ5. Both proteins were present in double-transfected extracts. Increasing the ratio ERβ to ERα resulted in decreased expression of ERα.

in the lysate, it was not expected to have any activity in the absence of any E_2. In the presence of 1 n*M* E_2, the observed total activity in the ERβ4 and ERα co-transfected cells was lower than expected based on their protein levels. At all the molar ratios of the proteins tested, the observed activities were close to what would be expected if only ERβ4 was present (Fig. 10, top right panel). These results show that ERα and ERβ4 heterodimers have lower activity than would be expected of the combined homodimers. Because ERβ4 is E_2 independent, the lower activity could be only due to inhibition of ERα when it dimerizes with ERβ4. The ERβ5 and ERα co-transfected lysates gave somewhat different results. The observed total activity in the absence of estrogen was 20–25% lower than expected activity of ERβ5 (Fig. 10, lower left panel), showing inhibition of E_2-independent activity of ERβ5 in the presence of ERα. We observed far lower activity in the ERα and ERβ5 co-transfected extracts than expected in the presence of estrogen (Fig. 10, lower right panel). A similar trend was seen in all the molar ratios tested. These results demonstrate that heterodimerization of ERβ5 with ERα results in inhibition of ERα in the presence of 1 n*M* estrogen.

Discussion

Since the first report by Moore et al. *(12)*, both ERβ4 and ERβ5 were considered to be non-functional short sequences spanning from the 3' end of exon 7 of ERβ1 with unique sequences in the place of exon 8. The data presented here established for the first time that, contrary to the previous report, ERβ4 and ERβ5 are full-length receptors. They have identical sequences with wild-type ERβ1, from the translational start site to the end of exon 7 and with unique sequences in exon 8. It is possible that Moore et al. *(12)* detected only the short sequences of these receptors previously because of mRNA degradation of their transcripts in testis from which they cloned their short sequences. A Blast search revealed that the distinct 10 bp coding sequence of ERβ5 in the place of exon 8 originated by retention of the 5' end of the intron between exon 7 and exon 8. The specific 16 bp coding sequence beyond exon 7 in ERβ4 was different from the sequences reported by Moore et al. *(12)*, and derived from the downstream a non-coding sequence of the ERβ gene (Fig. 2B). Upon transfection in COS-7 cells, both receptor plasmids generated the expected size proteins (Fig. 3).

Although ERβ4 and ERβ5 share sequence homologies with ERβ1 from exon 1–7, substitution of distinct sequences in the place of exon 8 resulted in unique properties. Unlike ERβ2, both receptors interacted with promoter sequences on DNA similar to ERα and ERβ1 (Fig. 5). When ERβ4 and ERβ5 were transfected in COS-7 cells, they predominantly localized in the nucleus (Fig. 6). This was expected as the nuclear localization signal was retained. Although they did not bind estrogen presumably due to truncation of exon 8 and substitution with short unique sequences in the place of exon 8 (Fig. 4), both receptors showed transcriptional activity in the absence of estrogen when co-trans-

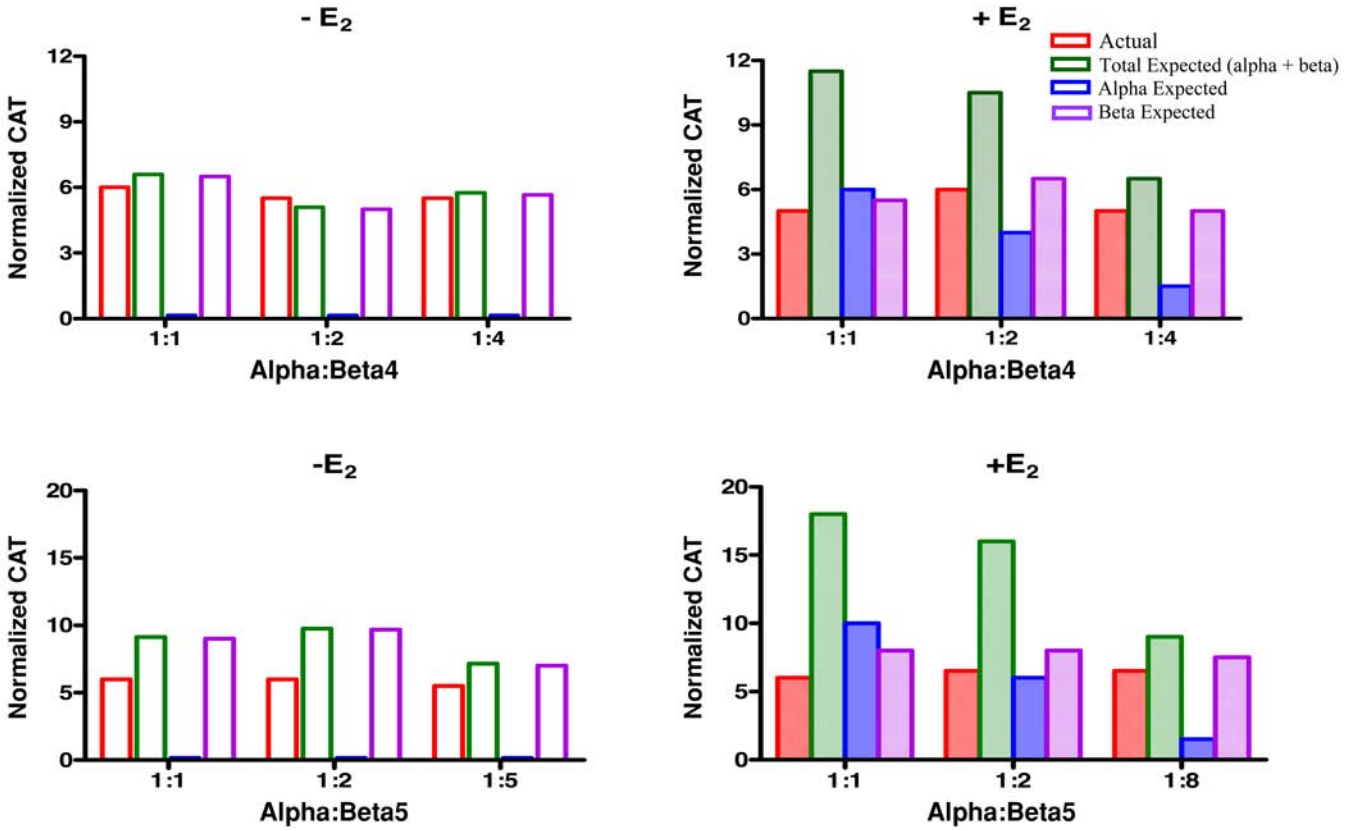

Fig. 10. CAT activities of lysates prepared from COS-7 cells transfected with ERα, p2ERE-TK-CAT and ERβ4 or ERβ5 plasmids. The lysates (50 μL), prepared as described above, were assayed for CAT levels using Roche CAT Elisa kit and the manufacturer's protocol. The observed total activity, expected total activity, and expected individual ERβ4, ERβ5, and ERα activities based on their protein expression levels in the lysates are shown. The CAT levels for lysates, which have varying molar ratios of ERα to ERβ4, and ERα to ERβ5 in the presence or absence of E_2, are shown as histograms. Top panels show histograms for lysates co-transfected with ERβ4 and ERα plasmids. In the absence of E_2, the observed activity was close to what was expected of ERβ4. ERα was not expected to have any activity under these conditions. In the presence of 1 n*M* E_2, the observed activity was close to what is expected of ERβ4 alone. Because ERβ4 is E_2-independent, the lower observed activity could be only due to inhibition of ERα. A similar trend was seen at all the molar ratios of ERα:ERβ4 tested. These data demonstrated the inhibition of ERα in the presence of ERβ4. Bottom panels show histograms for lysates transfected with ERβ5 and ERα plasmids. In the absence of E_2, the observed activity was less than what was expected of ERβ5, indicating inhibition of ERβ5 by ERα. In the presence of E_2, the observed activity was far less than what was expected from the combined activities. A similar trend was seen at all the molar ratios of ERα:ERβ5 tested. These data demonstrate that dimerization of these two receptors results in inhibition of ERα activity by ERβ5 in the presence of E_2.

fected with a CAT reporter gene containing ERE promoter sequences (Fig. 7). Stimulation with up to 1000 n*M* estrogen did not significantly increase the transcriptional activity of either receptor (Fig. 7). In similar experiments, ERβ2 did not show any transcriptional activity in agreement with the previous report by Ogawa et al. *(13)*. However, the rat ERβ2 seems to be different from human receptor in that it binds estrogen and exhibits transcriptional activity *(14)*. Both ERβ4 and ERβ5 differed from each other and from ERβ1 in their transcriptional activities in the absence of estrogen. The estrogen-independent ERβ5 activity was higher than ERβ4 and both were three to four times more active than ERβ1 (Fig. 7).

Because both receptors retain dimerization (C) domains, we next tested if they form heterodimers with ERα similar to ERβ1 and ERβ2 *(9)*, and modulate its activity. The results presented in Fig. 8 demonstrated that both receptors predominantly formed heterodimers with ERα. We did not observe any homodimers in either case, suggesting that these two receptors preferentially heterodimerize with ERα. To test whether ERα dimerization with ERβ4 or Eβ5 results in inhibition of its activity, we measured the transcriptional activity in cells doubly transfected with varying molar ratios of ERα and ERβ plasmids. The results presented in Fig. 10 demonstrate that heterodimerization of ERα with ERβ4 or with ERβ5 results in inhibition of ERα transcriptional activity in the presence of 1 n*M* estrogen. Our data also established that ERα partially inhibits E_2-independent transcriptional activity of ERβ5 (Fig. 10, lower left panel). These observations established, for the first time, that ERα modulates beta receptors.

The data presented here on ERβ4 and ERβ5 together with known properties of ERβ1, ERβ2, and ERβ3 establish that each of the five isoforms is distinct from the other in

ligand and DNA binding, and ERα interacting properties. The distinct functional and molecular properties of the five ERβ isoforms together with their tissue-specific expression levels *(15)* could play a pivotal role in tissue-specific actions of estrogens.

Materials and Methods

Omniscript reverse transcriptase kits and HotStart Taq PCR core kits, MinElute gel extraction kits, QIAprep Spin MiniPrep Kits, and Qiagen Plasmid Maxi kits were from QIAGEN Inc. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. RNAse inhibitor and random hexamers were from Applied Biosystems. PCR quality water and Tris–EDTA buffer were from BioWhittaker. Complementary DNA prepared from human ovary total RNA was available from previous studies *(16)*. The vectors, pCR®2.1-TOPO and pCR®3.1, Geneticin®, and Lipofectamine 2000 were obtained from Invitrogen. [2,4,6,7,16,17-^{3}H]-β estradiol, adenosine 5'-[γ-^{32}P] triphosphate, Enhanced Chemi Luminescence (ECL) reagents, Ready-To-Go T4 Polynucleotide Kinase kit, ProbeQuant™ G-50 Micro Columns, and POLY(dI-dC)·POLY (dI-dC) were from Amersham. Unlabeled ERE and GRE oligonucleotides and rabbit polyclonal antibodies against ERβ (H-150) and ERα (HC-20) were from Santa Cruz Biotechnology Inc. Horseraddish peroxidase (HRP)–conjugated goat anti-rabbit IgG, hydroxyapatite and protein molecular weight standards were from Bio-Rad. Charcoal/dextran–treated fetal bovine serum was from Hyclone. Trypsin–EDTA with and without phenol red, DMEM with and without phenol red, phenol red free Opti-MEM, antibiotic–antimycotic (penicillin–streptomycin and amphotericin) and DNA molecular weight standards were from Gibo-BRL Life Technologies. Protease-inhibitor cocktail containing AEBSF, EDTA, Bestatin, E-64 leupeptin, and aprotinin was from Sigma Chemical Company. COS-7 cells and MCF-7 cells were obtained from ATCC and maintained in DMEM media containing 10% fetal bovine serum and 1% antibiotic and antimycotic medium. CAT ELISA kit was from Roche Molecular Biochemicals. Monoclonal antibodies against ERβ (β14C8) were obtained from Gene-Tex. Full-length ERβ1 coding sequences cloned in the expression vector, pCXN2, was a gift from Ogawa and colleagues *(17)*, p2ERE-TK-chloramphenicol acetyl transferase (CAT) plasmid was a gift from Dr. Benita Katzenellenbogen, Univeristy of Illinois, Urbana. Full-length ERα cDNA sequence cloned in eukaryotic expression vector, pSG5, was available from previous studies *(18)*.

Amplification of Full-Length Coding Sequences for ERβ4 and ERβ5 by Asymmetric PCR

They were amplified from human ovary cDNA using a sense primer upstream of the ERβ1 translational initiation site, 5'GGTGTTTTCTCAGCTGTTAT3' (position, upstream of exon 1, bp 71–91) *(17)*, and isoform-specific anti-sense primers downstream of translational stop codons. The anti-sense primers for ERβ4, and ERβ5 were 5'GTCTGGGTTT TATATCGTCTGCAA3' (position, bp 294–271) *(12)* (Gen Bank accession no. AF061054) and 5'CACTTTTCCCAA ATCACTTCACC3' (position, bp 265–243) *(12)* (GenBank accession no. AF061055), respectively. For comparative purpose, ERβ1 and ERβ2 sequences were also amplified using specific anti-sense primers, 5'AGCACGTGGGCATTCA GC3' (position, exon 8, bp 1597–1580) *(17)* and 5'GTCAC TGCTCCATCGTTGCT3' (position, exon 8, bp 1907–1887) *(12)*, respectively. The PCRs were conducted in a total volume of 12.5 μL containing the cDNA reverse transcribed from 125 ng of total RNA from ovary, 1X PCR buffer, 1X Q solution, 200 μ*M* each of dNTPs, 1 μ*M* of anti-sense primer, varying concentrations (1 μ*M*, 2 μ*M*, 5 μ*M*, and 10 μ*M* or 1X, 2X, 5X, and 10X, respectively) of sense primer, and 0.6 U of HotStartTaq polymerase in an automatic thermal cycler (MJ Research) as previously described *(19,20)*. The PCR conditions were initial denaturation for 1 min at 95°C, followed by 94°C for 1 min, annealing for 1 min at 55°C, extension for 2 min at 72°C for 40 cycles, and final extension for 15 min at 72°C. PCR products (12.5 μL) were separated by electrophoresis in 1% Nu Sieve agarose gels in Tris–acetic acid–EDTA buffer and detected by ethidium bromide staining.

Cloning of Full-Length ERβ4 and ERβ5 PCR Products and Sequence Analysis

The ERβ4 and ERβ5 products obtained by asymmetric PCR were first cloned into pCR®TOPO 2.1-vector. Next, the full-length coding sequences were re-amplified using a sense primer that had Kozak sequence upstream of translational start codon, 5'GCCACCATGGATATAAAAAAC TCACCATCTAGC3' (position, upstream of exon 1, bp 99–125) *(17)*, and isoform-specific anti-sense primers as given above using their respective cloned pCR®2.1-TOPO vectors as templates. The amplified full-length sequences with Kozak sequences at the 5' end were cloned into an eukaryotic expression vector, pCR®3.1. A full-length ERβ2 coding sequence (bp 1512) was amplified by PCR from human ovary using specific anti-sense primer, 5'TCTAATCAAC TCGGTGGCCT3' (position, downstream of stop codon, bp 1910–1924), based on the published sequence *(12)* (accession no. AF051428) and cloned into pCR®3.1 expression vector as above. To deduce the nucleotide sequence of ERβ4 and ERβ5, the sense strands of both cloned plasmids were sequenced using a primer in the pCR®3.1 plasmid, 5'ATCCACTAGTCCAGTGTGGTGG3' (position, bp 706–727), and four forward primers that spanned from exon 1 through 6 of ERβ1. They were 5'CTTTAGTGGTCCATC GCCAGTT3' (position, exon 1, bp 361–382), 5'GTGTAC AATCGATAAAAACCGG3' (position, exon 3, bp 668–689), 5'GGCCTCCATGATGATGTCCCT3' (exon 4, bp 971–991), and 5'AGAATATCTCTGTGTCAA GGCC3' (exon 6, bp 1283–1304) *(17)*. Anti-sense strands of both cloned recep-

tors were sequenced with 5'ACCAGGGACT CTTTTGA GGTT3' (position, exon 1, bp 400–420), 5"TGGCAGCTCT TGCGCCGGTTTT3' (position, exon 3, bp 682–703), 5'TC CTTGTCGGCCAACTTGGTC 3' (position, exon 4, bp 992–1012), and 5'AATTGAGCAG GATCAT GGCC TT3' (exon 6, bp 1299–1320). In addition, they were sequenced with the above anti-sense primers that were applied to specifically amplify the ERβ4 and ERβ5. All the sequence analyses were performed by the cycle sequencing method on an automated DNA sequencer as described previously (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

Transfections of Plasmids into COS-7 Cells

Before transfections, all the plasmids were first tested for correct orientation and then re-precipitated under sterile conditions. For transient transfections, COS-7 cells were seeded into T-75 flasks and grown to 90–95% confluence. Three days before transfections, the cells were changed into phenol-red–free DMEM media supplemented with 10% charcoal–dextran–stripped fetal bovine sera without antibiotics. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, Lipofectamine 2000 (50 µL) was diluted to 1.5 mL with phenol-red–free Opti-MEM, and incubated at room temperature for 5 min. Purified plasmids (24 µg) were diluted to 1.5 mL with phenol-red–free Opti-MEM, added to the diluted Lipofectamine, and incubated for 30 min at room temperature. Before transfections, cells were washed twice with phenol-red–free DMEM supplemented with 10% charcoal–dextran–treated fetal bovine serum. The DNA–Lipofectamine complexes were added to the cells, mixed and left at 37°C in the CO_2 incubator. After 24–48 h, the cells were harvested using phenol-red–free Trypsin–EDTA. Stable transfectants were selected with Geneticin® (500 µg per mL of media). Whenever co-transfections of ER and p2ERE-TK-CAT plasmids were performed, 20 µg of ERE-CAT plasmid and 4 µg of ER plasmid were added to the cells. When two different ER plasmids were co-transfected with ERE-CAT plasmid, specified amounts of each ER plasmid was used.

Estrogen Binding Assays

COS-7 cells (80–90% confluent) were maintained for 3 d in estrogen-free medium, transfected with cloned ER pCR®3.1 plasmids for 24 h, and harvested using phenol-free Trypsin–EDTA. The whole-cell extracts were prepared by lysis on ice for 30 min in 20 m*M* HEPES, pH 7.6, 12% glycerol, 1 m*M* EDTA, 2 m*M* DTT, 400 m*M* KCl, and protease inhibitors and then six freeze–thaw cycles of freezing at –80°C and thawing on ice. The extracts were centrifuged at 27,000*g* in a Beckman Avanti refrigerated centrifuge for 20 min. The cell extracts were diluted to reduce the KCl concentration to 100 m*M* and used for estrogen-binding assays. The extracts (100 µg total protein) were incubated with 0–10 n*M* of [2,4,6,7,16,17-^{3}H]-β estradiol in the presence or absence of 200-fold excess of cold 17-β estradiol at 4°C for 16 h. The bound estradiol was separated from the free by adding hydroxylapitite slurry [100 µL of 50% (v/v)]. The hydroxyapatite-bound estradiol was measured by liquid scintillation counting. The results were analyzed by the method of Scatchard *(21)*.

DNA Binding Properties of ERβ4 and ERβ5 by Gel Mobility Shift Assay

A 27-bp double-stranded consensus ERE sequence, 5'G GATCTAGGTCACTGTGACCCCGGATC3', was labeled with [γ-^{32}P]ATP using Ready-To-Go T4 Polynucleotide Kinase kit according to the manufacturer's instructions. Briefly, 5 p*M* of ERE was incubated with 1 µL (10 µCi) of [γ-^{32}P]ATP in a total of 50 µL reaction buffer containing T4 polynucleotide kinase for 30 min at 37°C. The labeling reaction was stopped by adding 5 µL of 250 m*M* EDTA and the ^{32}P-labeled ERE was separated from the free [γ-^{32}P] ATP using ProbeQuant™ G-50 Micro Columns. For ERE binding, the transfected COS-7 cells or MCF-7 cells were lysed as above and the extracts were diluted to reduce the KCl concentration to 150 m*M*. The diluted lysates (20–30 µg of total protein) were first incubated with 1 µg of POLY (dI-dC) for 20 min at room temperature, then 1 µL of labeled ERE was added and incubated for an additional 30 min. The reaction mixtures were loaded onto pre-run 5% native polyacrylamide gels and electrophoresed at 200 mV for 90 min in 25 m*M* Tris–glycine pH 8.6 buffer containing 1 m*M* EDTA. The gels were dried and exposed to X-ray film. The experiments were also conducted in the presence of 3 p*M* cold ERE or GRE (5'AGAGGATCTGTACAGGATGTTC TAGAT3') where indicated.

Chloramphenicol Acetyl Transferase (CAT) Assays

To determine the transcriptional activity of ERβ4 and ERβ5, pC®73.1 plasmids containing their respective coding nucleotide sequences (4 µg) were co-transfected with CAT reporter gene containing ERE, p2ERE-TK-CAT plasmid (20 µg), into COS-7 cells that were maintained for 3 d in estrogen-free medium. After 24 h of transfection, cells were exposed to 0 n*M*, 100 n*M*, 500 n*M*, and 1000 n*M* estrogen for 24 h. The cells were harvested with phenol-red–free trypsin–EDTA and analyzed for CAT by ELISA using the reagents from Roche Diagnostics and the manufacturer's instructions. Briefly, cells were extracted with 1:5 diluted lysis buffer and used for CAT assays. The extracts (50–100 µg of total protein) in triplicate were added to ELISA plate wells that were coated with antibodies against CAT, incubated for 60 min at 37°C, and washed five times with wash buffer. Next, anti-CAT-DIG (200 µL) was added to the wells and incubated for 60 min at 37°C and then washed five times with wash buffer. This step was followed by the addition of anti-DIG peroxidase (200 µL), incubation for 60 min at 37°C, and washing five times with the wash buffer. Finally, peroxidase substrate (200 µL) was added, incubated for 40

min at room temperature, and the developed color read at 405 nm against a reference of 490 nm in a Dynatech ELISA reader (5000L Series). A standard curve was generated every time an assay was performed using the standard CAT supplied with the reagents.

Intracellular Localization of ERβ4 and ERβ5 Proteins

COS-7 cells were seeded into slide flasks, transfected with ERβ plasmids for 24–48 h, and fixed in 4% paraformaldehyde in PBS for 1 h and washed with PBS. Double immunofluorescence labeling was carried out with monoclonal and polyclonal antibodies specific to ERβ as follows: After a brief immersion (15 min) in cold (–20°C) acetone: methanol (1:1 v/v) for 15 min, the cells were washed three times with PBS containing 0.02% Tween 20 (10 min each wash). Cells were incubated in a 1:100 dilution of polyclonal antibodies against N-terminal region of ERβ (H-150) in PBS for 3 h at room temperature, washed in PBS containing 0.02% Tween-20 for 30 min (three washes, 10 min each) followed by 3% normal goat serum in PBS for 60 min at room temperature to block any nonspecific binding of the secondary antibody. Starting from this step, incubations were carried out in a dark room with a small lamp and a red light bulb to minimize fading of the fluorescence. The cells were incubated in a 1:100 dilution of secondary antibody [goat anti-rabbit IgG (H + L) conjugated to Alexa Fluor 488] in PBS for 1 h at room temperature. Cells were washed in PBS with 0.02% Tween-20 for 30 min (three washes, 10 min each). This was followed by incubation in 1:100 dilution of monoclonal antibodies against ERβ (β14C8) in PBS for 3 h at room temperature. Cells were washed in PBS containing 0.02% Tween-20 for 30 min (three washes, 10 min each) and incubated in a 1:100 dilution of secondary antibody [goat anti-mouse IgG (Fc Specific) TRITC conjugate, Sigma T-6653] in PBS for 1 h at room temperature. Cells were washed in PBS–Tween-20 for 30 min (three washes, 10 min each) and mounted in glycerol/*p*-phenylenediamine to minimize fading and visualized with confocal microscope (carried out at the Microscopy Facility, University of Alberta).

Electrophoresis, Western Blotting, and Other Methods

Protein estimations were carried out by the method of Lowry *(22)* as modified by Bensadoun and Weinstein *(23)*. Protein gel electrophoresis and Western blotting were performed as described previously *(24)*. SDS-PAGE (15%) was conducted in a Bio-Rad slab gel apparatus as described by Laemmli *(25)*. Proteins were transblotted to nitrocellulose membranes as described by Towbin et al. *(26)*. Blocking and antibody treatments were performed as previously described *(27)*. A dilution of 1:200 anti-ERβ polyclonal antibodies (H-150) was used to probe ERβ4 and ERβ5 proteins on nitrocellulose blots. A dilution of 1:500 anti-ERα (HC-20) polyclonal antibodies were used to probe for ERα protein. The antigen–antibody complexes were detected using a 1:5000 dilution of the HRP-conjugated goat anti-rabbit IgG and development with the ECL detection system.

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