Putative functional characteristics of human estrogen receptor-beta isoforms

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Abstract

Estrogen receptors (ERα and ERβ) are clearly multifaceted in terms of structure and function. Several relatively abundant ERβ isoforms have been identified, which can be differentially expressed in various tissues. In order to provide insight into the possible role of the ERβ family in breast tissue a study of the putative functions of the human (h) ERβ1, hERβ2 and hERβ5 isoforms was undertaken. Only hERβ1 was found to bind ligand, which induced conformational changes as determined by protease digestion assays. All ERβ isoforms could bind to and bend DNA although the relative efficiency with which they bound DNA differed with hERα>hERβ1>hERβ2>>hERβ5. All ERβ isoforms inhibited ERα transcriptional activity on an estrogen-response element (ERE)-reporter gene. The relative activities were hERβ1>hERβ2 >hERβ5; however, only hERβ1 had transcriptional activity of its own. Both LY117018–hERα and LY117018–hERβ complexes alone could activate transcription on a TGF-β3-CAT gene. Although hERβ2 and hERβ5 had no activity alone, they inhibited hERα but not hERβ1 transcriptional activity of transforming growth factor (TGF)-β3-CAT. In marked contrast to activity on an ERE-CAT reporter gene, hERβ1 did not modulate ERα transcriptional activity on a TGF-β3-CAT reporter gene. These data support promoter-specific differential activities of hERβ isoforms with respect to models of ERα regulated gene expression, and suggest that they may have a role in differentially modulating estrogen action.

Journal of Molecular Endocrinology (2003) 30, 13–29

Introduction

The estrogen receptor family of steroid hormone receptors is clearly multifaceted (Hall et al. 2001) and more complex than originally thought. There are two genes which encode estrogen receptors (ER), ERα and ERβ. Both are ligand regulated transcription factors which classically modulate target gene transcription by binding as homo- and/or heterodimers to estrogen responsive sequences in target gene promoters (Cowley et al. 1997). These receptors likely have distinct roles in estrogen action, independent of each other when they are expressed separately (Couse & Korach 1999) but can also have direct interactions due to heterodimerization when the receptors are expressed together in the same target cell (Enmark et al. 1997). In addition, both ERs may encode variant isoforms generated by alternative splicing mechanisms (Lu et al. 1998, Moore et al. 1998). In particular there are data to support variant isoforms of ERβ at the protein level (Fuqua et al. 1999, Fujimura et al. 2001). Furthermore, we have shown in human breast tissues that variant forms of ERβ are more abundant than the wild-type at least at the RNA level (Leygue et al. 1999).

Human (h) ERβ2 (also called hERβcx (Ogawa et al. 1998b)) and ERβ5 variant mRNAs are missing the wild-type exon 8 sequences and contain extra sequences which are distinct from each other, followed by sequences that are then identical with each other (see Fig. 1). They are predicted to encode C-terminally truncated ERβ-like proteins identical to wild-type until amino acid residue 468 (by reference to the long form of hERβ1) (Ogawa et al. 1998a). After amino acid 468 hERβ2 is predicted to encode 28 novel amino acids, with the full-length protein having a predicted molecular mass of 55.5 kDa. In contrast, amino acid 468 hERβ5 is predicted to encode only 5 novel
a long form of 530 amino acids (Leygue et al. 1998a; Ogawa et al. 1998b), human ERβ2 (pcDNA3-1 hERβ2, long form (Ogawa et al. 1998b)) and human ERβ5 (pcDNA3-1 hERβ5, long form) proteins were synthesized by in vitro transcription–translation as described above. Ligand binding studies were conducted as previously described (Lu et al. 2000). In vitro-generated receptor was diluted 10-fold in buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred microliters of the diluted protein were used in each binding reaction that contained varying concentrations of [3H]E2 (0.01–100 nM), followed by overnight incubation at 4 °C. Non-specific binding was determined by parallel incubations containing a 200-fold excess of unlabeled E2. Unbound steroid was removed by addition of 500 µl 0·5% charcoal–0·05% dextran in the above dilution buffer for 30 min at 4 °C followed by centrifugation at 10 000 × g for 10 min at 4 °C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total [3H]E2 solutions using a scintillation counter. The ratio of specifically bound/unbound steroid and the concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, $K_d$.

Limited proteolytic digestion analysis

Conformational studies were performed as described previously (Beekman et al. 1993). In vitro-synthesized ERs were incubated with agonists (E2, diethylstilbestrol) and antagonists (4-OH-TAM, LY117018) overnight at 4 °C. The liganded receptors were then diluted 1:10 (v/v) in TE buffer, then 20 µl of this ER solution were treated with increased concentrations of trypsin (0·2 to 5 µg) for 20 min at room temperature and stopped by the addition of loading buffer. The samples were boiled and were analyzed directly by SDS-polyacrylamide gel electrophoresis (10% w/v). The gel was dried and the digested bands were visualized by autoradiography.

Electrophoretic mobility gel-shift assay (EMSA)

In vitro-synthesized human ERs were used for EMSA. Typically 1 µl programmed lysates containing equal amounts of each receptor as determined

Materials and methods

Materials

17β-Estradiol (E2), 4-hydroxytamoxifen (4-OH-TAM) and CAPS (3-cyclohexylamino-1-propanesulfonic acid) were from Sigma Chemical Co. (St Louis, MO, USA). ICI 182,780 was a gift from Dr A E Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, UK). LY117018 was a gift from Eli Lilly Co. (Indianapolis, IN, USA). [3H]17β-Estradiol, [14C]chloramphenicol, and [35S]-methionine were from New England Nuclear (Boston, MA, USA). [α-32P]dCTP, [γ-32P]ATP, and [35S]-ATP were from ICN Pharmaceuticals (Irvine, CA, USA). All cell culture reagents were obtained from GIBCO/BRL (Burlington, Ontario, Canada).

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (Tnt coupled Reticulocyte Lysate System; Promega, Madison, WI, USA). Reactions were performed according to the manufacturer’s instructions.

Scatchard analysis

Human ERα (pcDNA3-1/wild-type human ERα from HEGO (Green et al. 1986)), human ERβ1 (pcDNA3-1 hERβ1, long form of 530 amino acids (Leygue et al. 1998a; Ogawa et al. 1998b), human ERβ2 (pcDNA3-1 hERβ2, long form (Ogawa et al. 1998b)) and human ERβ5 (pcDNA3-1 hERβ5, long form) proteins were synthesized by in vitro transcription–translation as described above. Ligand binding studies were conducted as previously described (Lu et al. 2000). In vitro-generated receptor was diluted 10-fold in buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred microliters of the diluted protein were used in each binding reaction that contained varying concentrations of [3H]E2 (0.01–100 nM), followed by overnight incubation at 4 °C. Non-specific binding was determined by parallel incubations containing a 200-fold excess of unlabeled E2. Unbound steroid was removed by addition of 500 µl 0·5% charcoal–0·05% dextran in the above dilution buffer for 30 min at 4 °C followed by centrifugation at 10 000 × g for 10 min at 4 °C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total [3H]E2 solutions using a scintillation counter. The ratio of specifically bound/unbound steroid and the concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, $K_d$.

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Electrophoretic mobility gel-shift assay (EMSA)

In vitro-synthesized human ERs were used for EMSA. Typically 1 µl programmed lysates containing equal amounts of each receptor as determined
by polyacrylamide gel electrophoresis of $^{35}$S-methionine-labeled protein generated in parallel in vitro transcription–translation assays, was assayed in EMSA. One microliter lysate was incubated in a final volume of 20 µl, and the reaction solution was 5 mM Tris-HCl, pH 7·5, 100 mM KCl, 2 mM dithiothreitol, 5% v/v glycerol and contained 2 µg polyd(I-C). The binding reaction was initiated by adding 1 µl (approx. 10 fmol) 5′-[$^{32}$P]-end-labeled, double stranded estrogen-response element (ERE) oligonucleotide (35 mer, 5′-AAGTTTTGATCAGGTCAGTGTGACCTGACTTTGGAC-3′ containing the vitellogenin A2 ERE sequence), and the mix was incubated at 20°C for 30 min. DNA-bound complexes were electrophoretically separated on a 4·5% loosely cross-linked acrylamide gel (1:29 bis:acrylamide) at 150 V for 90 min at 20 °C in 0·5× TBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1 µg ER antibody (usually 1 µl H222 for ERα, or 1 µl PAI-310 for ERβ1 and ERβ2) were run to determine the presence of super-shifted antibody-bound ER–ERE complexes (data not shown). Reticulocyte lysates containing in vitro-translated ER proteins were incubated with or without saturating concentrations of ligand (estrogen or antiestrogen) at 4 °C overnight to allow receptors to bind ligand, followed by EMSA.

**DNA bending assay**

The DNA bending vector ERE Bend I (kindly provided by Dr A Nardulli, University of Illinois, Urbana, IL, USA) (Nardulli & Shapiro 1992) was digested with EcoRI and EcoRV to produce a 430 bp DNA fragment with a single consensus ERE either at the end (EcoRI fragment) or in the middle (EcoRV fragment). The fragments were then gel purified, labeled by incubation with polynucleotide kinase in the presence of

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**Figure 1** (A) cDNA structure of human ERβ1 (hER-β1), human ERβ2 (hER-β2) and human ERβ5 (hER-β5) and predicted proteins of the human ERβ isoform cDNAs. Genomic structure of the human estrogen receptor β locus on chromosome 14. Human ERβ cDNA, expressed sequence tags and published promoter sequences (accession numbers in the text) were aligned with sequences from two genomic clones of human chromosome 14 (AL162756/CNS01 RHJ and AF215937). Exons 1C to 1H are found only in one cDNA (accession number AB006589). aa, amino acids.
Cells, cell culture and transient transfection

For transient transfection analysis, tagged ER expression vectors were generated. Human ERα, ERβ1, ERβ2 and ERβ5 were tagged at their N-terminus with a polyhistidine and an Xpress epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc., Burlington, Ontario, Canada). Cos-1 and Cos-7 cells were obtained from the ATCC (Manassas, VA, USA). The cells were routinely cultured in DMEM containing 5% v/v fetal calf serum (FBS), 1% w/v glucose, glutamine and penicillin–streptomycin (5%CM). To obtain estrogen-depleted cells, the culture medium of stock cells was changed to phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, 1% w/v glucose, glutamine and penicillin–streptomycin (5%CS) and replaced every 2 days. Six days later the medium was replaced by 10%CS until required for experiments. For transient transfection experiments, the cells were transfected using the Effectene transfection reagent according to the manufacturer’s instruction (QIAGEN, Mississauga, Ontario, Canada). Briefly, the day before transfection, the estrogen-depleted cells were seeded in 6-well plates at 2.5 × 10^5 cells per well in 2 ml 5%CS and left overnight. The plates were 70–80% confluent on the day of transfection. The transfection mixture was prepared according to the manufacturer’s protocol, then fresh medium (5%CS) was added to the transfection mixture and 0.6 ml per well of the above mixture with either ERE-II-TCO-CAT (a gift from P Webb (Webb et al. 1995)) or transforming growth factor (TGF)-β3-CAT-reporter plasmid DNA (Yang et al. 1996) was added. ER expression plasmid (50–450 ng) or empty vector and 100 ng β-gal pCH110 plasmid DNA (Pharmacia Canada, Mississauga, Ontario, Canada) was added drop-wise into the medium (CS) and the plates were gently swirled to ensure uniform distribution of the DNA–Effectene complexes. Vehicle (ethanol), estradiol-17β or LY117018 was then added 20 to 30 min later. The cells were left for 48 h and then harvested. Cell extracts were prepared by freeze/thawing and were used to determine chloramphenicol acetyl transferase (CAT) and β-galactosidase activity as previously described (Dotzlaw et al. 1992).

Western blot analysis

For Western blot analysis, 2.5 × 10^5 Cos-1 cells were set up in 6-well plates, then transiently transfected with plasmids and treated with estrogen or antiestrogen under the same conditions as for the CAT assay described above. Cells were harvested 48 h after transfection, washed once with Isoton II and then the washed cell pellets were resuspended in 200 µl Isoton II. Aliquots of cell suspension (150 µl) were extracted and used for Western blots and the remainder was used for determination of β-galactosidase activity. For Western blotting, the cells were pelleted and then extracted using 40 µl hot (95 °C) extraction buffer J with shaking for 20 min at 95 °C as previously described by Joel et al. (1998). The entire extract was subjected to 10% SDS-polyacrylamide gel electrophoresis as previously described (Adeyinka et al. 2002). The separated proteins were transferred to nitrocellulose membranes and processed as previously described (Adeyinka et al. 2002). Detection of the tagged estrogen receptor proteins was by incubation of blots with anti-Xpress antibody (1/5000 in TBST, Cat#R910-25, Invitrogen Canada Inc.) overnight at 4 °C, followed by washing and incubation with secondary antibody (horseradish peroxidase conjugated goat anti-mouse antibody, 1/5000 in TBST, Jackson Immuno Research Labs Inc., West Grove, PA, USA) at room temperature for 2 h. Visualization was carried out using the SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

Statistical analysis

Differences in variance were tested using ANOVA, where appropriate. Differences between individual mean values were then determined using Student’s t-tests. All tests were performed using GraphPad Prism statistical analysis software (GraphPad Software Inc., San Diego, CA, USA).
Results

Identification and organization of hERβ1, hERβ2 and hERβ5 cDNA sequences at the hERβ locus on chromosome 14

The estrogen receptor beta gene has been localized to human chromosome 14q22–24 and the genomic structure of 8 exons comprising hERβ1 has previously been published (Enmark et al. 1997). However, the previously described variant hERβ2 (also called hERβcx) and hERβ5 mRNA contain only sequences corresponding to exons 1 to 7 of hERβ1 and then they diverge (see Fig. 1A). They do not contain exon 8 sequences of hERβ1, but contain sequences termed exon 9, which are located downstream of exon 8 on chromosome 14 (Fig. 1B), identified using database sequences of chromosome 14 (accession numbers CNSO1 RHJ and AF215937) and the Human Genome Working Draft. It should be noted that neither of these genomic sequences contain an extra A 5’ of the start site of translation for hERβ, that would place another upstream ATG in frame with the known coding region and introduce 18 amino acids to the N-terminal of the known coding region, as recently described (Wilkinson et al. 2002). Interestingly, hERβ5 mRNA also contains sequences between exon 7 and part of exon 9 which are not present in either hERβ1 or hERβ2 mRNA. These hERβ5 mRNA specific sequences can be found immediately following exon 7 sequences in intron 7 of the human ERβ gene (Fig. 1B), suggesting that the normal splice donor site is not recognized and a cryptic splice donor site is present in intron 7. Furthermore the exon 9 sequences present in hERβ5 cDNA start 28 nucleotides downstream of those present in hERβ2, suggesting a cryptic splice acceptor site is present within exon 9. There are also multiple non-coding exons 5’ to exon 1 as previously identified (Enmark et al. 1997), since several hERβ cDNAs contain sequences in their 5’ UTR which are found further upstream of the previously described exon 1 on chromosome 14 (Fig. 1B; 1H–1C seen in AB006589, 1B seen in NM_001437, AX234638, AF05428, AF060555, AB006589 references). The sequences of a recently characterized promoter region of hERβ (Li et al. 2000) are found immediately upstream and overlapping with exon 1B. However, the presence of hERβ mRNAs whose 5’UTR contain exonic sequences found upstream of this documented promoter suggest that there are alternative promoters for the hERβ gene. This is similar to the hERα gene and suggests that regulation of expression of these genes is complex (Kos et al. 2001).

The predicted open reading frames for hERβ1, hERβ2 and hERβ5 are shown in Fig. 1A. hERβ2 contains amino acids 1–468 which are identical to hERβ1; the sequence then diverges containing another 28 novel amino acids encoded in the open reading frame. hERβ5 was isolated as a partial cDNA but is likely also to be identical to hERβ1 from amino acids 1–468 and then diverges containing another 5 novel amino acids. Both these variant hERβ proteins would be truncated at the C-terminus, disrupted in helix 11 and missing helix 12 and therefore unlikely to bind ligand or have AF2-mediated transcriptional activity. Lack of ligand binding has been confirmed using in vitro generation of these proteins as outlined below.

Ligand binding activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Human ERβ1 has previously been shown (Enmark et al. 1997) to bind E2 with high affinity and specificity, and our data confirm these findings. Figure 2A shows specific saturable binding of [3H]E2 to in vitro translated hERβ1 with a calculated $K_d=0.11$ nM. However, the open reading frames of hERβ2 and hERβ5 cDNA predict for C-terminally truncated proteins compared with hERβ1 and are predicted not to bind ligand. As shown in Fig. 2B and C no saturable binding of [3H]E2 to in vitro-translated hERβ2 or β5 was observed.

Human estrogen receptor isoform conformational status and ligand induced changes

To determine the possible conformational status of variant hERβ isoforms, a previously used limited trypsin digestion assay (Beekman et al. 1993, McDonnell et al. 1995) was employed to compare the proteolytic digestion patterns of variant 35S-methionine labeled hERβ, hERβ1, hERβ2 and hERβ5 in the presence and absence of estrogens and antiestrogens (4-OH-TAM and LY117018). The results are shown in Fig. 3. In the absence of any ligand, all ER isoforms were sensitive to
proteolysis. In the presence of estradiol both hER\(\alpha\) and hER\(\beta_1\) become more resistant to digestion, and a 32.5 kDa resistant band (shown by asterisks in Fig. 3) was observed. In contrast addition of the antiestrogens 4-OH-TAM and LY117018 did not significantly affect the sensitivity of the receptors to trypsin compared with the receptors in the absence of ligand. The sensitivity of the variant isoforms hER\(\beta_2\) and hER\(\beta_5\) was not affected by ligand, consistent with their inability to bind ligand and suggesting that the variant isoforms are unlikely to be in an activated conformation.

**DNA binding and bending activity of hER\(\beta_1\) and variant isoforms hER\(\beta_2\) and hER\(\beta_5\) proteins**

Similar amounts of each recombinantly produced ER isoform protein, determined as described in the Materials and methods section, were used in the electrophoretic mobility shift assays. As previously demonstrated hER\(\beta_1\) and hER\(\beta_2\) can bind to an ERE in a gel mobility shift assay (Fig. 4A), although the efficiency of hER\(\beta_2\) DNA binding was less than hER\(\beta_1\) (Moore et al. 1998). In contrast, Ogawa et al. (1998b) showed no DNA binding activity for hER\(\beta_2\). hER\(\beta_5\) also has the ability to bind an ERE in gel mobility shift assays (Fig. 4A), but was less efficient than hER\(\beta_2\). The specificity of the binding was determined by competition with excess unlabeled ERE whereas no competition was seen with an excess of unlabeled nonspecific 33 mer oligonucleotide.

DNA bending assays demonstrated that hER\(\alpha\), hER\(\beta_1\) and hER\(\beta_2\) were all able to bend DNA as demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison with the mobility of complexes when the ERE is at the end (E) of the DNA fragment (Fig. 4B and C; Nardulli & Shapiro 1993, Lu et al. 2000). The calculated bending angle for hER\(\alpha\) was 64.8 ± 1 (mean ± s.e.m., \(n=3\)), for hER\(\beta_1\) it was 53.6 ± 0.5 and for hER\(\beta_2\) it was 54.6 ± 0.7. A lower overall signal of the retarded complexes was seen with hER\(\beta_1\) and hER\(\beta_2\) compared with hER\(\alpha\) which likely reflects the lower efficiency of the hER\(\beta\) isoforms of binding to an ERE compared with the hER\(\alpha\). Furthermore, the DNA binding ability of hER\(\beta_5\) was too low to obtain accurate data for DNA bending calculations. No effect of ligand was observed (data not shown).

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**Figure 2** Determination of estradiol binding to (A) hER\(\beta_1\), (B) hER\(\beta_2\), and (C) hER\(\beta_5\). Increasing amounts of \(^{3}H\)estradiol-17\(\beta\) (0.01–100 nM) were incubated with a constant amount of each in vitro-transcribed/translated hER\(\beta\) protein. Two separate experiments were performed. Total binding is shown by the closed circles and non-specific binding is shown by the open circles.
Figure 3 Sensitivity of human estrogen receptor isoforms to protease digestion. Radiolabeled ER was made in vitro as described in the Materials and methods section, and digested with increasing levels of trypsin, with and without ligand (E₂, 4-OH-TAM or LY117018 (LY)). The products were visualized by autoradiography after SDS-PAGE. Resistant bands are shown by arrows. The asterisks show the agonist induced resistant 32.5 kDa bands.
Transcriptional activity of hERβ1, hERβ2 and hERβ5

The ability of tagged ERs to activate transcription was initially investigated using Cos-1 cells and an ERE containing reporter gene, ERE<sub>452</sub>-delta-TCO-CAT, which has two vitellogenin A2 (−333/−288) EREs upstream of a CAT reporter (Webb et al. 1995). Epitope tagged receptors were used so that relative expression of all the ERs could be measured using antibodies to the epitope tag, and preliminary experiments demonstrated that the tagged ERα and ERβ1 were similar to their untagged counterparts in activating transcription with and without ligand (data not shown). Preliminary studies showed that transfection of

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Shift bands

Free EREs

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Shift bands

Free ERE

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Shift bands

Free ERE
50 ng ERα expression vector gave maximal estradiol-induced transactivation of this reporter gene. hERα and hERβ1 activated transription in a ligand inducible manner (Fig. 5), but hERβ1 was overall less active than hERα (P<0.0001, n=5), and increased expression of hERβ1 did not alter this relationship. These data are consistent with previous findings. As shown in Fig. 5, low doses of estradiol (0-1 nM) which significantly activated hERα did not activate hERβ1 (P<0.0001, n=5), and the apparent ligand-independent activity (zero ligand added) of hERα was significantly higher than that of hERβ1 (P=0.024, n=5). This ligand-independent activity was inhibited by 0.1 and 100 nM of the antiestrogen LY117018 (a raloxifene analog) as well as by hERβ1 (50–450 ng) and hERβ2 (450 ng) (data not shown). These results are not due to over-expression of hERβ1 protein relative to hERα, since under conditions where similar levels of hERα protein (50 ng hERα expression plasmid; see Fig. 6, lane 4) and hERβ1 are expressed (150 ng hERα expression plasmid; see Fig. 6, lane 4) the ligand-independent activity of the receptors is still significantly different (see Fig. 5, compare histogram bar 1 with 7) and the estrogen-inducible (0.1 and 100 nM) activity of hERβ1 (see Fig. 5, compare histogram bars 2 and 3 with histogram bars 8 and 9) is not further increased. The expression of the variant isoforms hERβ2 and hERβ5 alone demonstrated little if any transcriptional activity under these conditions (Fig. 5).

ERα and hERβ isoforms can heterodimerize (Cowley et al. 1997), which may underlie the functional interactions between ER isoforms. All hERβ isoforms tested inhibited the transcriptional activity of hERα on an ERE containing promoter (Fig. 7A and B) but the various hERβ isoforms had different efficiencies with hERβ1 > hERβ2 > hERβ5. Ligand activation of hERβ1 did not affect its ability to decrease the activity of hERα, since under conditions when it was not activated (0 or 0.1 nM estradiol, see Fig. 5) hERβ1 activity was similar to that under conditions when it was activated (100 nM estradiol). Variant isoforms of hERβ had little if any effect on hERβ1 activity on ERE-containing promoters (data not shown).

The transcriptional activity of ER isoforms was next examined on the non-ERE-containing promoter, TGFβ-3-CAT, where the DNA binding domain of ERα is not required for activity (Yang et al. 1996). This promoter was shown to be preferentially activated by the raloxifene-bound hERα compared with estradiol in cultured cells (Yang et al. 1996), and we have previously shown differential abilities of murine ERβ isoforms to affect this promoter compared with ERE-containing promoters (Lu et al. 2000). Therefore, the activity of hERβ isoforms on TGFβ-3-CAT was examined (Fig. 8). Optimal activity for hERα was obtained with transfection of 50 ng expression plasmid (data not shown). A significant increase in transcription was obtained with 0.1 nM LY117018 that was not further increased with 100 nM LY117018 treatment (P=0.0061, n=3). LY117018 significantly increased the transcriptional activity of hERβ1 on the TGFβ-3-CAT reporter gene at the lower levels of hERβ1 expression (50 ng, P=0.008; 150 ng, P=0.02, n=3) but at high levels of hERβ1 expression (450 ng), a significant increase in ligand-independent activity was seen, and no further increase was seen due to ligand. Overall, hERβ1 was significantly less active than hERα in inducing TGFβ-3-CAT (P<0.0001, n=3).

Figure 4 (A) Determination of the ability of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) to bind to DNA. Autoradiograph of an electrophoretic mobility gel shift analysis of in vitro-transcribed/translated hERα, hERβ1, hERβ2 and hERβ5 proteins binding to a 35 mer double stranded ERE oligonucleotide containing the vitellogenin A2 ERE sequence. Free ERE and the shifted complexes are indicated. The presence of the appropriate ER isoform in the shifted complex was determined by the ability of a specific antibody (h222 for ERα, PA1 for the ERβ proteins) to super-shift the complex (data not shown). Specificity of the complexes was determined by the activity of a 200-fold excess of the unlabeled ERE (specific competitor) to compete for the shifted complex and non-specific interactions were determined using a 200-fold excess of unlabeled nonspecific 33 mer oligonucleotide (nonspecific competitor). (B and C) Comparison of the ability of (B) hERα (hERα) and hERβ1 (hERβ1) and (C) hERβ1 and hERβ2 (hERβ2) to bend DNA. In vitro-transcribed/translated ER isoforms were preincubated with 10 nM estradiol-17β followed by incubation with radiolabeled ERE. Bend fragments as described in the Materials and methods and were subjected to electrophoretic gel mobility shift analysis. DNA bending was demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison to the mobility of complexes when the ERE is at the end of the DNA fragment (E).
Although there was a trend towards inhibition of TGFβ-3-CAT with increasing expression of hERβ1 (data not shown), this was not statistically significant.

When the ability of hERβ isoforms to affect hERα activity was investigated at the TGF-β3 promoter, differences between the wild-type and variant isoforms were observed. The wild-type hERβ1 did not significantly affect hERα transcriptional activity at any level of expression tested (Fig. 9A and B). However, under the same conditions hERβ2 significantly inhibited hERα transcriptional activity on TGFβ-3-CAT (P=0.0002, n=3), and as expected the effect was not influenced by LY117018, since hERβ2 does not bind ligand. However, hERβ2 inhibits both the ligand activated and the non-ligand activated (data not shown) hERα (P=0.017, n=3) at the TGFβ-3-CAT promoter. hERβ5 also inhibited hERα transcriptional activity on TGFβ-3-CAT but only at the highest expression of hERβ5 (Fig. 9B, P=0.038, n=3). Similar to their action at an ERE-containing promoter, the truncated ERβ variants ERβ2 and ERβ5 do not modulate wild-type hERβ1 transcriptional activity on TGFβ-3-CAT (data not shown).

### Discussion

There is a growing body of evidence that ERα and ERβ can be expressed together in some cell types and independently expressed in others (Dotzlaw et al. 1997, Jarvinen et al. 2000, Saji et al. 2000). If expressed together they form heterodimers, which under experimental conditions are preferred over homodimerization (Cowley et al. 1997). Further, transient coexpression of ERα and ERβ in cell lines results in ERβ1-induced reduction of ERα activity.

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<th>E2 (nM)</th>
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**Figure 5** Transcriptional activity of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) on ERE regulated CAT reporter gene. Increasing amounts of hER isoform expression vectors were co-transfected with an ERE-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 h with or without estradiol-17β. Results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency) ± S.E.M. of 5 independent experiments for hERα, hERβ1 and hERβ2, and 3 independent experiments for hERβ5. See text for statistical analysis.
at low ligand concentrations, as measured using ERE-regulated reporters (Hall & McDonnell 1999). A conclusion from these data is that ERβ can directly modulate ERα activity. This has significance since many reports exist of differential expression of the two receptors under conditions of altered estrogen sensitivity. For example, ERβ expression is significantly downregulated and ERα expression upregulated during human breast tumorigenesis, suggesting that ERβ’s ability to modulate ERα is significantly altered during breast tumorigenesis (Leygue et al. 1998b, Roger et al. 2001). In addition, current data show that in normal and neoplastic breast tissues, the level of expression of the C-terminally truncated ERβ variants, ERβ2 and ERβ5, is markedly higher than the ligand binding ERβ1. These data suggest that the variant ERβ isoforms may also have a role in modulating estrogen and possibly antiestrogen action in human breast cells. The experiments described in this manuscript were undertaken to gain insight into the possible role of the truncated ERβ variants.

Our data show that only hERβ1 is able to bind ligand. Steroid hormone receptors are known to undergo conformational changes during the process of activation especially due to ligand binding, and differences are seen between agonist and antagonist binding (Beekman et al. 1993, McDonnell et al. 1995). Recent structural analyses of the ligand binding domain (LBD) of several nuclear receptors suggest that the LBD contains common structural motifs that generate a conserved ligand binding pocket, and that agonists and antagonists bind to the same site but induce different conformational changes that are now known to affect transcriptional function, providing structural evidence for antagonism (Brzozowski et al. 1997). The variant hERβ isoforms, while not binding ligand, may exist in an activated state in the absence and presence of ligand; however, our data suggest that hERβ2 and hERβ5 are unlikely to be in an activated conformation, and this is consistent with their inability to activate transcription of either a ‘classical’ or a ‘non-classical’ estrogen receptor regulated reporter gene.

All ERβ isoforms examined (ERβ1, ERβ2, and ERβ5) inhibit the transcriptional activity of ERα on ERE-containing promoters, while only ERβ1 has any activity alone. This confirms and extends previous data and demonstrates that the relative inhibitory activity of the ERβ isoforms is...
Figure 7 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from an ERE (vitellogenin A2) regulated CAT reporter gene in the presence and absence of ligand following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.
This correlates with the relative efficiencies with which ER homodimers bind to DNA and may suggest a competition of the beta isoform homodimers with ER homodimers for DNA binding. However, since heterodimers are preferred under these conditions, it is likely that these predominate under our experimental conditions and the intrinsically lower transcriptional activity of the heterodimers are predominant. Cowley et al. (1997) demonstrated that when hERα and hERβ1 are expressed at both a 1:1 and 1:2 ratio the ERα/ERβ1 heterodimer was predominant. This heterodimer had a DNA binding affinity similar to that of the ERα homodimer, and was capable of recruiting steroid receptor coactivator-1 (SRC-1). However, the heterodimer has less transcriptional activity than the ERα/ERα homodimer, suggesting that it may be less efficient in recruiting coactivators than the ERα homodimer. In contrast, the C-terminally truncated hERβ2 has markedly reduced ability to bind to DNA and likely the ERα/ERβ2 heterodimer also binds less well than ERα/ERα homodimers to an ERE (Moore et al. 1998, Ogawa et al. 1998b). But in contrast to hERβ1, hERβ2 does not recruit coactivators (Ogawa et al. 1998b). Our data show that hERβ5 is less efficient than hERβ2 in binding to DNA, and is also unlikely to recruit coactivators. However, at an ERE the wild-type hERβ1 is more potent than either of the two variants in inhibiting the ability of ERα to activate transcription. So it appears that the inability to
Putative functions of the ERβ isoforms
recruit coactivators is not correlated with the ability of ERβ isoforms to inhibit ERα activity. Since DNA activity is also a reflection of efficiency of dimerization, it is speculated that the truncated ERβ isoforms have reduced ability to dimerize with ERα and form stable heterodimers than the wild-type ERβ1. Together with our Western blot data it seems that significant inhibition of ERα transcription occurs at levels of ERβ1 expression that are less than or equivalent to ERα (50 ng ERβ1 plasmid makes less protein than 50 ng ERα plasmid, but still significantly affects ERα transcription activity). Therefore our data would be consistent with the mechanism of inhibition being related to a high efficiency of dimerization and reduced efficiency in recruiting coactivators, but not the inability to recruit coactivators.

Interestingly, marked differences in the ability of the ERβ isoforms to affect ERα activity are seen at an estrogen receptor responsive site where the mechanism of transcriptional regulation is quite distinct from that operating at a classical ERE, e.g. the so-called raloxifene responsive element in the TGF-β3 promoter (Yang et al. 1996). This is in marked contrast to the results seen at an ERE regulated reporter gene. The ER responsive site in the TGF-β3 promoter is poorly activated by the estradiol–ERα complex, but is strongly activated by the raloxifene–ERα complex. In addition, the DNA binding domain of the ER is not required for this activation. It is assumed that protein–protein interactions between ERα and other transcription factors bound to this promoter are involved in regulation. However, the identity of these ‘other’ transcription factors is unknown. Using an analog of raloxifene, LY117018 (Lu et al. 2000), we have confirmed that this promoter is poorly activated by the estradiol–ERα complex (and this was not altered in our hands by treatment of the transfected Cos-1 cells with epidermal growth factor (Lu & Giguere 2001); data not shown) but was significantly activated by the LY117018–ERα complex. Similarly, the LY117018–hERβ1 complex was found to activate transcription from the TGF-β3 promoter, but in contrast to the murine ERβ1 (Lu et al. 2000), is less active than the LY117018–hERα complex. Human ERβ2 and hERβ5 alone could not activate this promoter. This is in contrast to the murine ERβ2 variant (Lu et al. 2000) which is structurally quite different to the hERβ2. Furthermore, no murine equivalent to either hERβ2 or hERβ5 isoforms, that are frequently expressed in human tissues, has as yet been identified. However, coexpression of increasing amounts of hERβ2 and hERβ5 with ERα resulted in inhibition of LY117018–ERα transcriptional activity but not LY117018–ERβ1 activity from the TGF-β3 promoter. In contrast to an ERE-containing promoter is the observation that the wild-type hERβ1 did not significantly inhibit the transcriptional activity of the LY117018–ERα complex at the TGF-β3 promoter. At this promoter the differences in the hERβ isoform activity on LY117018–ERα complexes were correlated to the ability to recruit coregulatory factors. Significant effects of hERβ2 on hERα were seen under conditions of equimolar expression, as determined by Western blot analysis of the similarly tagged proteins, but hERβ5 was less active than hERβ2 and this is consistent with a reduced efficiency of dimerization. There appears to be differential expression of hERβ isoforms at least at the RNA level in different human tissues, as well as altered relative expression during breast tumorigenesis (Leygue et al. 1999, Omoto et al. 2002), and altered levels of hERβcx (hERβ2) as well as hERβ1 during prostate cancer progression (Fujimura et al. 2001). Therefore, it is possible that the differential activities of hERβ isoforms on some genes may have both physiological and pathophysiological importance.

In conclusion we have characterized some potential functions of several commonly expressed hERβ isoforms. Generally, the ligand binding wild-type hERβ1 has transcriptional activity alone on both ‘classical’ and ‘non-classical’ estrogen responsive promoters, although it is less efficient than ERα. Furthermore, the hERβ family of receptors generally negatively modulate ERα activity.

**Figure 9** Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from a TGF-β3-CAT reporter gene in the presence and absence of the raloxifene analog LY117018 following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)± S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.
transcriptional activity when coexpressed at ‘classical’ as well as ‘non-classical’ ER responsive promoters. However, promoter specific differential activity of the various hERβ isoforms was found, in particular between the wild-type hERβ1 and its C-terminally truncated variants hERβ2 and hERβ5. The possibility that there is differential expression of the hERβ isoforms suggests that they may have a role in differentially modulating estrogen action.

Acknowledgements

This research is supported by the Canadian Breast Cancer Research Initiative. E L holds a career development award (BC000577) from the USAMRMC.

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Received in final form 24 October 2002
Accepted 30 October 2002