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Estrogen receptor β acts as a dominant regulator of estrogen signaling

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The physiological effects of estrogens are mediated by two intracellular transcription factors, the estrogen receptors (ERs), that regulate transcription of target genes through binding to specific DNA target sequences. Here we describe alterations in cellular responses to different ER agonists and to the anti-estrogenic compound tamoxifen resulting from co-expression of the two ERs in transient co-transfection experiments. Our results demonstrate that ERβ can act as a negative or positive dominant regulator of ER activity. This is manifested through reduced transcriptional activity at low concentrations of estradiol (E_2); increased antagonistic effects of tamoxifen on E_2 stimulated activity; and enhanced agonistic action of the phytoestrogenic compound genistein. Furthermore, using chimeric proteins lacking the N-terminal activation function 1 (AF-1), we show that the differential responses of ERα and ERβ to different agonists and antagonists are primarily dictated by inherent differences in the C-terminal ligand-binding domains of the receptors, whereas the magnitude of transcriptional activity is influenced by ERα AF-1, but not ERβ AF-1. The ERα AF-1 activity appears to be modulated upon co-expression of both ERs. The alterations in transcriptional activity resulting from co-expression of ERα and ERβ are probably due to the formation of α/β heterodimeric complexes. This study demonstrates that co-localization and subsequent heterodimerization of ERα and ERβ may result in receptor activity distinct from that of ER homodimers. *Oncogene* (2000) **19,** 4970–4978.

Keywords: ERα; ERβ; heterodimerization; agonists; antagonists

Introduction

The estrogen receptors (ERs) belong to a group of ligand-inducible transcription factors known as the nuclear receptor superfamily (Laudet *et al*., 1992). Members of the family share several functional and structural similarities such as a variable N-terminus containing a transactivation function (AF-1), a centrally located DNA binding domain (DBD) consisting of two highly conserved zinc finger motifs and, C-terminally of the DBD, a region involved in binding of ligand, dimerization and transactivation, referred to as the ligand binding domain (LBD) (Gronemeyer and Laudet, 1995). The nuclear receptor family includes receptors for estrogens, progestins, androgens, glucocorticoids, mineralocorticoids (Beato *et al*., 1996), thyroid hormone, fatty acids and vitamin D3. The majority of nuclear receptor family members consists of a steadily growing number of receptors for which the ligand is unknown, the so called orphan receptors (Mangelsdorf and Evans, 1995).

Estrogens have long been recognized to be critical for the development, maturation and function of the female reproductive system. Lately the role of estrogens in heart and vascular protection and for maintenance of bone mass has also attracted considerable interest (Farhat *et al*., 1996). Studies of ERα knock-out mice have revealed important functions for estrogens also in the male reproductive system (Hess *et al*., 1997; Lubahn *et al*., 1993). Adversely, estrogens have been implicated in development and progression of tumors in breast and endometrium (Ciocca and Fanelli, 1997). Because of the estrogen-dependent growth of many breast tumors, endocrine therapy with anti-estrogens such as tamoxifen is often successful. However, some tumors become resistant to anti-estrogenic treatment possibly reflecting changes in ER status within the tumor (Jordan and Morrow, 1999).

The identification of a second estrogen receptor has added a new dimension to the complexity of estrogen signaling (Kuiper *et al*., 1996). The new receptor was named ERβ to distinguish it from the previous one, consequently named ERα. Subsequently, human and mouse homologs of ERβ were isolated (Mosselman *et al*., 1996; Pettersson *et al*., 1997; Tremblay *et al*., 1997). ERβ displays extensive sequence similarity to ERα, most notable in the DBD where the amino acid identity is 97% whereas the LBD shows an overall 55% similarity to ERα. However, the ligand binding pocket and the ligand dependent activation function (AF-2) show a higher degree of resemblance. Functionally both receptors share several characteristics in that they bind estradiol (E_2) and related compounds and recognize and initiate transcription from palindromic DNA sequences referred to as estrogen response elements (ERE) (Kuiper *et al*., 1996). ERβ has been reported to have weaker transcriptional activity in response to E_2 compared to ERα in transient co-transfection assays in several cell systems (Cowley *et al*., 1997; Pettersson *et al*., 1997; Watanabe *et al*., 1997). ERβ also, in contrast to ERα, fails to show agonistic response to the well known anti-estrogen tamoxifen (Tremblay *et al*., 1997; Watanabe *et al*., 1997). Recently ERα, but not ERβ, was demonstrated to bind to and activate transcription from the DNA core element known as an SF1 (Steroidogenic Factor 1) response element, thus demonstrating functional differences between the ERs also at the DNA binding level (Vanacker *et al*., 1999).

The N-terminally located A/B domain, which contains the ligand independent transactivation function AF-1, is poorly conserved between the ERs. The activity of ERα AF-1 has been described as highly cell

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specific, with varying activity depending on cell type (Katzenellenbogen *et al*., 1996). Studies using receptor chimeras where the A/B domains of the ERs were interchanged have demonstrated that differences in transcriptional potency as well as response to anti-estrogens rely on the nature of this domain. The activity of the respective AF-1 and AF-2 of the ERs was also found to be influenced by cellular context (McInerney *et al*., 1998). We have previously demonstrated the formation of functional heterodimeric complexes between the two ER subtypes (Pettersson *et al*., 1997). The importance of heterodimerization as well as several aspects of ERβ function are still unclear. A recent study, using mutant ERs with altered DNA binding specificity which allowed measurement of transcriptional activity induced exclusively by α/β heterodimers, demonstrated that while two functional AF-2's were required for transcriptional activity, activation could occur if only one of the ER subunits within the heterodimer was bound by E_2, albeit to a lower extent (Tremblay *et al*., 1999).

Given the reported differences between ERα and ERβ we wanted to examine functional aspects of co-expression in the presence of receptor agonists and antagonists. Alterations in transcriptional activity resulting from ERα/ERβ co-expression were analysed using transient co-transfection assays under carefully monitored conditions.

Results

Co-expression of ERα and ERβ negatively influences transcriptional response to low concentrations of E_2

We transiently transfected the human embryonic kidney cell line 293 with a luciferase reporter construct containing two copies of a consensus ERE inserted in front of a minimal thymidine kinase promoter together with expression plasmids encoding human ERα or mouse ERβ. The cells were treated with increasing concentrations of 17β-estradiol (E_2) for 24 h, harvested and the reporter activity was determined. Maximal reporter activity was obtained within the range of 5–30 ng of both ERα or ERβ encoding plasmids respectively (data not shown) under the conditions used, suggesting similar expression levels of each receptor as judged from their transcriptional activity. All subsequent co-expression experiments were carried out within these optimal limits to exclude uncontrollable events such as auto-squelching. ERβ consistently induced reporter activity to a lesser degree compared to ERα, reaching approximately 40%, which is in agreement with previous work using the same cell line (Pettersson *et al*., 1997, Figure 1c, left hand panel and data not shown).

In agreement with previous observations, ERβ is poorly activated by E_2 concentrations below 1 nM (Figure 1b) (Barkhem *et al*., 1998). In contrast, ERα, reaches approximately 50% activation already at 0.01 nM and shows maximal transcriptional activity at 0.1 nM (Figure 1a). The poor response of ERβ to low concentrations of E_2 was also observed in HeLa and HepG2 cells and is therefore not restricted to the 293 cell line (data not shown). In co-expression experiments, the amount of the ERα-expressing plasmid was kept constant at 10 ng (a concentration within the time limits of maximal transcriptional activity) and increasing quantities of the ERβ-expressing plasmid were added in an α : β ratio of 1 : 0.5, 1 : 1 and 1 : 1.5, respectively (5, 10 and 15 ng, equaling a total of 15, 20 and 25 ng, all within the limits of maximal response). Intriguingly, co-expression of ERα and ERβ results in a shift in transcriptional potential (Figure 1c). The maximal magnitude of activity was reminiscent of that of ERα homodimers, but the E_2 concentration required to obtain full activity was shifted in an ERβ-dose dependent manner to nanomolar concentrations (compare gray bars of each panel in Figure 1c). The decrease in transcriptional activity observed at low E_2-concentrations is not due to over-expression of ERs as we also performed the same series of transfections using increasing amounts of ERα-plasmid instead of ERβ without detecting any reduction in transcriptional response (Figure 1d). Moreover, the reduction in reporter gene activity only occurred under conditions where ERβ was demonstrated to be poorly activated; at 1 nM E_2 full transcriptional activity was obtained, regardless of the amount of co-transfected ERβ (Figure 1c, compare black bars).

Co-expression of ERβ with ERα increases the antagonistic effect of tamoxifen

Tamoxifen, an antiestrogenic compound clinically used in endocrine therapy of breast cancer, has long been recognized as a cell and tissue type dependent mixed agonist/antagonist for ERα (Jordan and Morrow, 1999; Katzenellenbogen *et al*., 1996). Both ERs bind tamoxifen with comparable affinity but a lack of agonistic effect of tamoxifen on ERβ has been reported, suggesting a functional difference between the two receptor subtypes (Tremblay *et al*., 1997; Watanabe *et al*., 1997).

The ability of tamoxifen to antagonize E_2-induced activity of both ERs was assessed in 293 cells transiently transfected as previously described, and treated with 1 nM E_2 alone, or in combination with increasing concentrations of tamoxifen. As evident from the results shown in Figure 2b tamoxifen is a more potent antagonist of the E_2-induced activity of ERβ compared to ERα (Figure 2a). Whereas the ligand-induced activity of ERβ is inhibited already at 100 nM tamoxifen, this concentration has only a minor antagonistic effect on the transcriptional activity of ERα, and not even 1 μM tamoxifen can completely antagonize the E_2-induced activity of ERα. This is in agreement with the described non-agonistic effect of tamoxifen on the ERβ subtype. The pronounced anti-estrogenic effect of tamoxifen on ERβ homodimers suggested that ERα/ERβ heterodimers may be more strongly antagonized by tamoxifen than ERα homodimers.

To test this hypothesis we studied the effect of co-expression of both ERs under the same conditions as in Figure 1c. The maximal level of activation was not altered when the amount of ERα was kept constant, but the presence of ERβ induced an increased antagonistic effect of tamoxifen in a dose-dependent manner, whereas the response to E_2 alone was intact at all concentrations of co-expressed ERβ (Figure 2c,

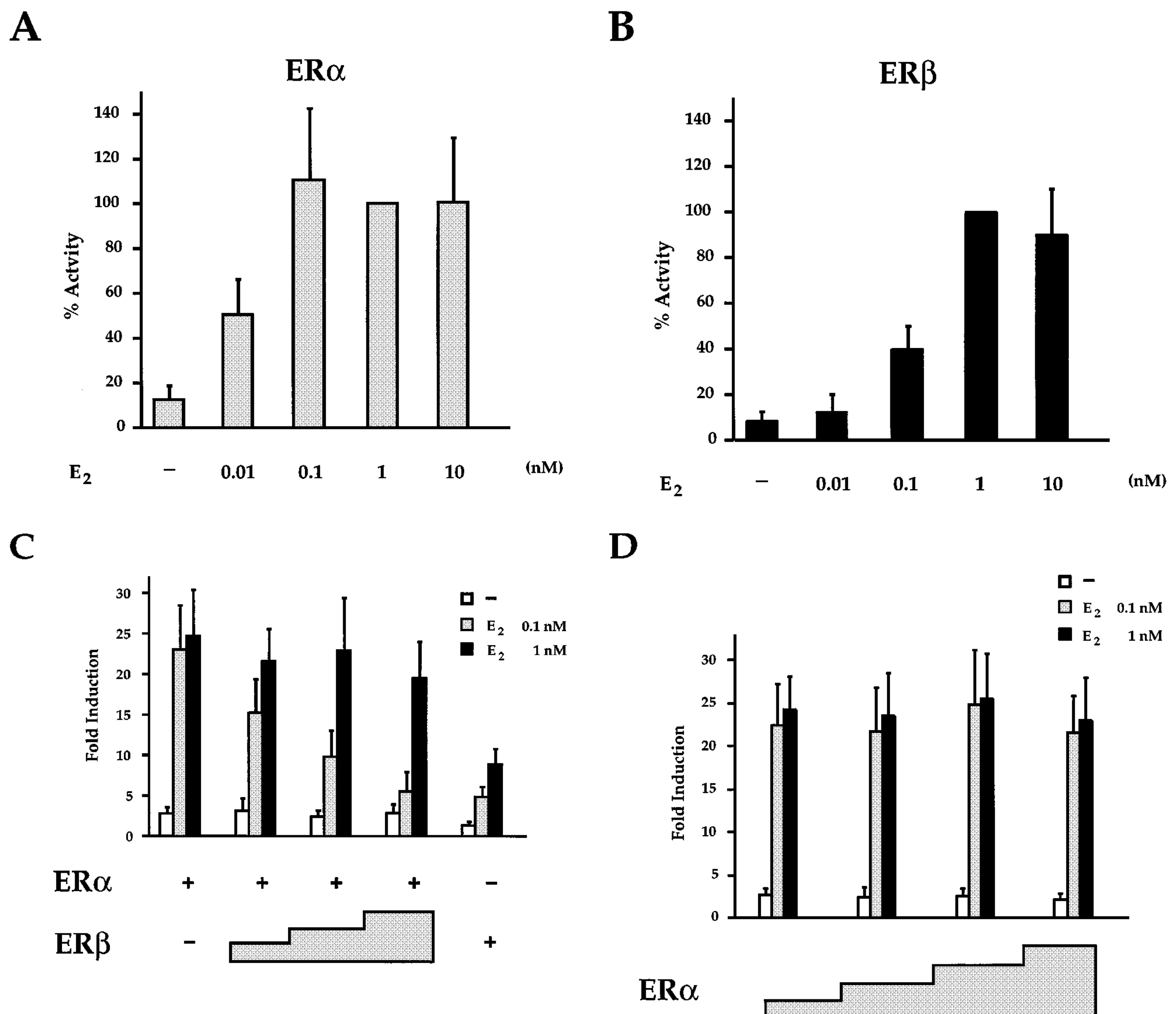

Figure 1 Higher concentrations of E_2 is required for efficient transcriptional activity in the presence of ERβ alone or ERβ co-expressed with ERα. 293 cells were transiently transfected with a luciferase reporter gene construct containing two copies of a consensus ERE and expression plasmids for ERα or ERβ. The transcriptional response to different concentrations of E_2 by ERα (**a**, gray bars) or ERβ (**b**, black bars) was determined. Data is presented as % activity ± s.d., where reporter activity obtained at 1 nM of E_2 was arbitrarily set to 100 for each receptor. All experiments were done in duplicates at least three times. (**c**) Effects of co-expression of ERβ together with ERα on response to E_2. Ratio of expression plasmids α : β were 1 : 0.5, 1 : 1, or 1 : 1.5 (panels 2, 3, and 4, respectively). (**d**) Control experiment using increasing amount of ERα expression plasmid under the same conditions as in **b**. Data is presented as mean of fold induction ± s.d. from at least three independent experiments performed in duplicates. Activity of reporter plasmid alone without hormone treatment was arbitrarily set to 1

compare gray bars to black and striped bars respectively). Increasing the concentration of transfected ERα, instead of ERβ did not result in any change of tamoxifen antagonism (data not shown).

ERβ promotes the agonistic effect of genistein

Given the observations that ERβ negatively influenced the transcriptional activity in cells where ERα and ERβ were co-expressed at low E_2 concentrations or in the presence of tamoxifen, we wanted to investigate whether ERα would reciprocally inhibit transcriptional activity in the presence of an ERβ selective ligand. Previous studies have indicated that the isoflavonoid genistein shows a higher affinity for ERβ (Barkhem *et al.*, 1998; Kuiper *et al.*, 1997). 293 cells were co-transfected as before with the ERE-reporter plasmid and ERα or ERβ expressing plasmids and treated with increasing amounts of genistein. The results were compared to reporter activity at 1 nM E_2 (Figure 3a,b). At 20 nM genistein, ERβ reaches approximately 80% of the activity obtained at 1 nM E_2 (Figure 3b). In contrast, ERα shows almost no activity at this concentration (Figure 3a). Both receptors are fully active at 200 nM of genistein as judged by comparison with E_2. Genistein acts as a super-agonist for both ER subtypes at 2 μM resulting in more than 200% of maximal activity obtained with E_2, which could however be due to the ability of genistein at high concentrations to modulate protein kinase signaling pathways that may alter the phosphorylation status of the ERs. These observations are inconsistent with a study carried out with 293 cells stably expressing ERα or ERβ where genistein was described as acting as a partial agonist in the ERβ expressing cells albeit with a selective affinity for the β receptor (Barkhelm *et al.*,

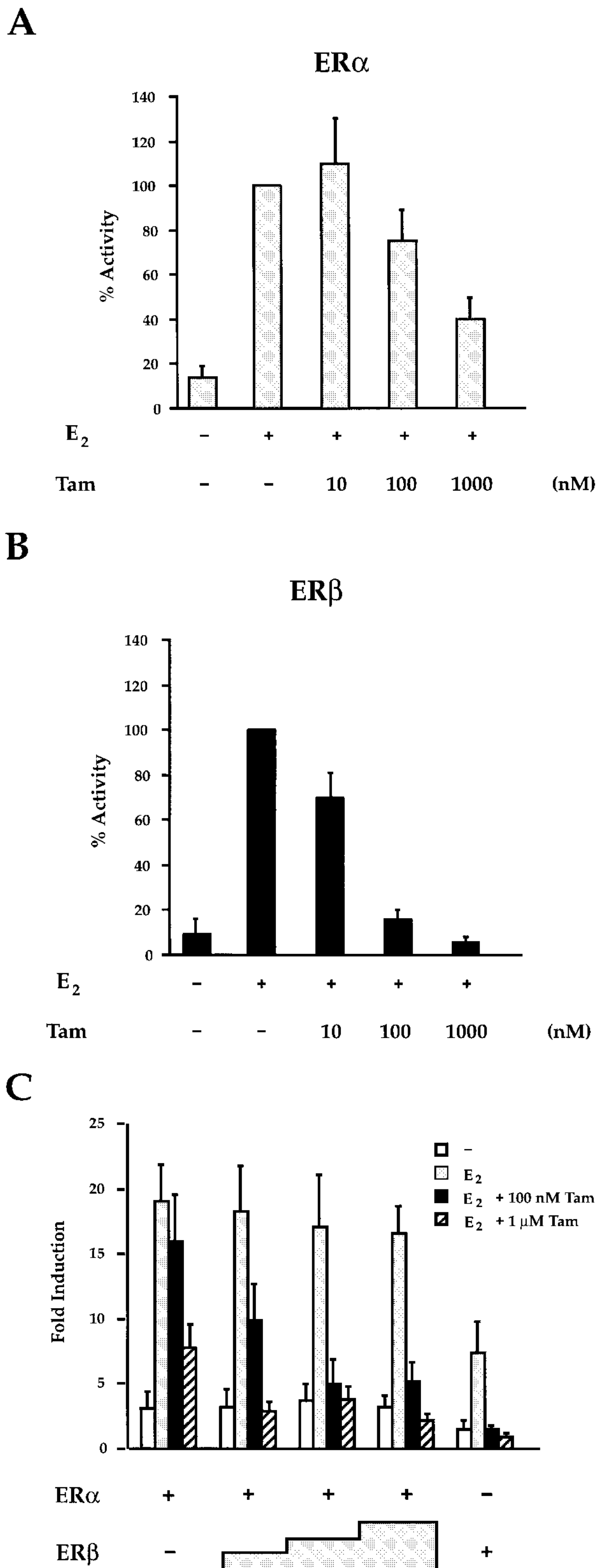

Figure 2 ERα/ERβ co-expression enhances the antagonistic effects of tamoxifen. 293 cells co-transfected with the ERE-luciferase reporter and ERα and/or ERβ expressing plasmids as described in Figure 1, were treated with increasing concentrations of tamoxifen in combination with 1 nM E_2. The effects of tamoxifen in combination with E_2 on the transcriptional activity of ERα (**a**) or ERβ homodimers (**b**) or co-expressed ERα/ERβ (**c**) were assessed. Data is presented as % activity±s.d., where reporter activity at 1 nM of E_2 was arbitrarily set to 100 for each receptor (**a** and **b**), or as mean of fold induction±s.d., where activity of reporter plasmid alone with no hormone treatment was arbitrarily set to 1 (**c**). Data represents experiments performed in duplicates at least three times

1998). In the mentioned study the cells were treated with genistein for 72 h, whereas we measured activity after 24 h, which could account for the observed discrepancy.

We next performed co-expression experiments with both ERs in the presence of genistein (Figure 3c). Surprisingly, the reporter activity at 20 nM genistein was higher when both receptors were co-expressed than what was observed with either receptor alone. The activity of ERα alone at this concentration is marginally above basal levels but clearly ERα contributes to the increase in transcriptional activity, since the reporter activity is well above that conveyed by the ERβ homodimer (Figure 3c, compare gray bars of central panels with left and right hand panels). To exclude that this observation was due to increased amounts of ERβ, we performed a corresponding experiment in the absence of ERα without detecting any alterations in response to 20 nM of genistein (Figure 3d).

Differential responses to ER agonists and tamoxifen, but not transcriptional magnitude, are primarily mediated by the AF-2

Recent studies performed by us and others indicate functional differences between the two ER subtypes. Comparisons between the amino acid sequences of the different functional domains reveal almost identical DNA binding domains, striking resemblance in helix 12 and E_2-binding regions of the LBD, but low degree of conservation in the A/B domain containing the ligand independent AF-1 and in the less well characterized hinge domain positioned between the DBD and the LBD. The AF-1 of ERα has been demonstrated both to act independently in the absence of ligand, but also cooperatively together with the ligand activated AF-2 (Berry *et al*., 1990; Tzukerman *et al*., 1994). A recent study using A/B domain substituted ER chimeras supported previous observations that agonistic response of ERα to tamoxifen is relying on the AF-1 (McInerney *et al*., 1998). To be able to distinguish between the activity of AF-1 and AF-2 of the two ER subtypes, we generated chimeric receptors where the hinge domain (D), the LBD (E) and the F domain of ERα and ERβ respectively, were linked to the DBD of the yeast factor Gal4 (Figure 4a). These constructs allowed studies of the activity of the AF-2 of each ER subtype without interference from the AF-1. The Gal4-αDEF and Gal4-βDEF chimeras were co-transfected together with a luciferase reporter gene construct containing Gal4 binding-sites into 293 cells which were subsequently treated with different concentrations of E_2 (Figure 4b). In contrast to the wild type receptors, the ERα chimera showed only a slightly more potent transcriptional response compared to the ERβ chimera. However Gal4-βDEF, in analogy to wild type ERβ was poorly activated by E_2 levels below 1 nM (Figure 4b, compare light gray bars and black bars). Co-expression of both receptor chimeras in 1 : 1 ratio resulted in a reduced response to 0.1 nM E_2 compared to Gal4-αDEF.

When tamoxifen was used together with E_2, the activity of Gal4-βDEF was antagonized at lower concentrations of tamoxifen compared to Gal4-αDEF, resembling the results obtained with the full length

receptors in the same cellular context (Figure 4c, compare black bars of most left hand panel with most right hand panel). Moreover, 1 μM, but not 100 nM, tamoxifen more strongly antagonized the activity of the Gal4-αDEF in comparison to wild type ERα (compare striped and black bars of the left hand panel of Figure 4b with Figure 2a). Co-expression of Gal4-αDEF and Gal4-βDEF resulted in intermediate reporter activity at 100 nM tamoxifen. Finally, identical experiments were carried out in the presence of increasing concentrations of genistein (Figure 4d, cells treated with 1 nM of E_2 were included to enable comparison of reporter activity levels). Gal4-βDEF induced reporter activity at 20 nM of genistein whereas Gal4-αDEF did not, similar to what was observed with the full length receptors, thus demonstrating that the selective response to low concentrations of genistein is determined by the ERβ-LBD.

Co-expression of both chimeric proteins resulted in an intermediate response at 20 nM genistein clearly contrasting to the enhanced activity obtained with full length receptors (Figure 3c), which indicates that the response to 20 nM genistein by co-expressed ERs involves the N-terminal parts of the receptors.

Discussion

Heterodimerization between steroid receptors represents a relatively recently discovered phenomenon. Heterodimerization between the glucocorticoid and mineralocorticoid receptors has been suggested to contribute to tissue specific actions of glucocorticoids (Trapp *et al.*, 1994). In addition, the androgen receptor and the glucocorticoid receptor have been shown to mutually interfere with each other's functions through the formation of heterodimeric complexes (Chen *et al.*, 1997). The ERs have been demonstrated to form functional heterodimeric complexes in several studies, but the consequences of ER

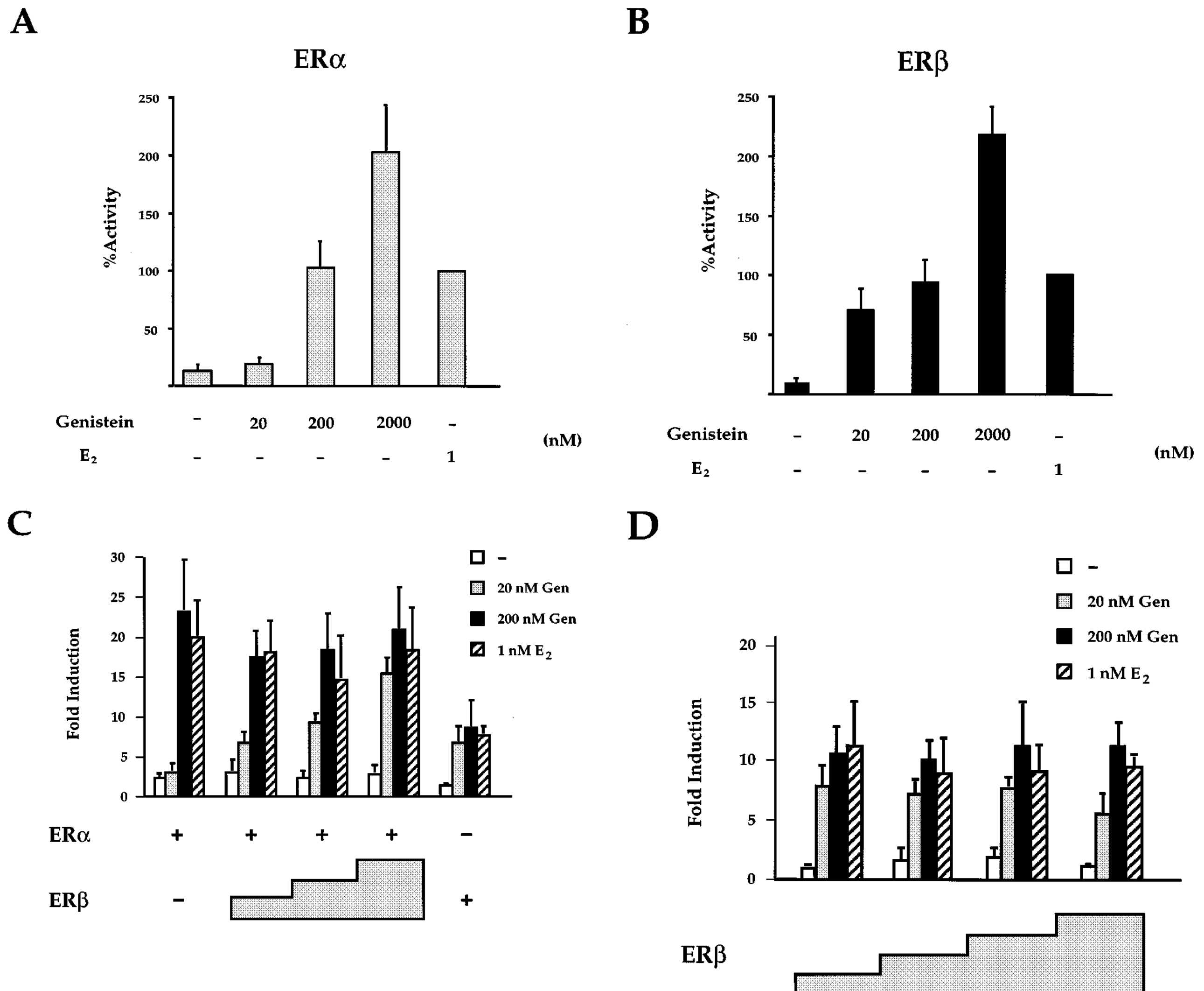

Figure 3 Co-expression of ERα and ERβ enhances the agonistic effect of genistein. 293 cells were co-transfected with the ERE-Luciferase reporter and ERα and ERβ expressing plasmids. Increasing concentrations of genistein were added to the cells. Transcriptional activity of ERα and ERβ alone (**a** and **b**, respectively) or ERα and ERβ co-expressed (**c**) were measured. Increasing amount of ERβ expression vector under the same conditions as in **c** as a control experiment (**d**). 1 nM of E_2 was included as a comparative control in all experiments. Data is presented as % activity±s.d., where reporter activity obtained at 1 nM of E_2 was arbitrarily set to 100 for each receptor (**a** and **b**), or as mean of fold induction±s.d., where activity of reporter plasmid alone without hormone treatment was arbitrarily set to 1 (**c** and **d**). Each set of transfections were performed in duplicates at least three times

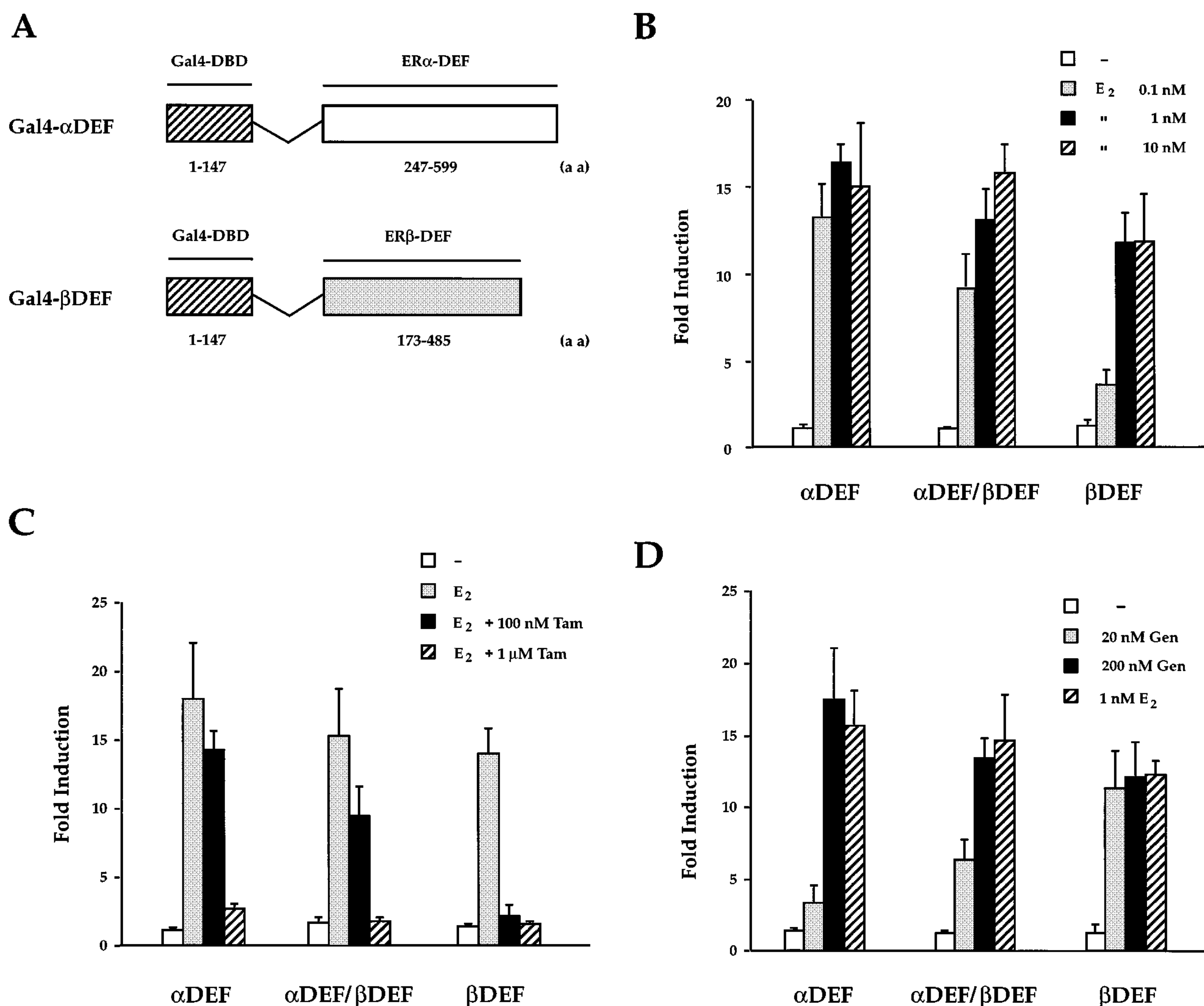

Figure 4 Differential responses to E_2, tamoxifen and genistein, but not transcriptional potency, are primarily dictated by the DEF regions of ERα and ERβ. A luciferase reporter construct containing Gal4 binding sites was transiently transfected into 293 cells together with plasmids expressing the Gal4-DBD chimeric proteins schematically presented in (**a**). Cells were treated with increasing concentrations of E_2 (**b**), tamoxifen in combination with 1 nM E_2 (**c**) or genistein (**d**). Data is in each case presented as fold induction±s.d. of at least three independent experiments performed in duplicate. Activity obtained with reporter plasmid alone without hormone treatment was arbitrarily set to 1

heterodimerization on estrogen signaling have not been determined.

The observations presented here demonstrate that co-expression of ERs influences cellular responses to both agonists and antagonists. ERβ displays a weaker transcriptional potency in this cellular context compared to ERα, and is shown to require higher concentrations of E_2 for full activity. Co-expression experiments demonstrate that ERβ has the ability to inhibit the activity of ERα at E_2 concentrations below the ERβ activity threshold level. This probably occurs as a consequence of the formation of heterodimeric complexes between ERα and ERβ. Alternatively, the changes in reporter activity could result from transcriptional interference of the respective homodimers. However, it is difficult to envision that ERβ homodimers which are not transcriptionally active at E_2 levels below 1 nM, would still be able to compete more efficiently for binding to the ERE-containing reporter than ligand-activated ERα homodimers.

Furthermore, we show that co-expression of ERα and ERβ, and treatment with tamoxifen increase the antagonistic effect in an ERβ dose-dependent manner. Again, ER heterodimerization appears to be the most logical mechanism to explain these observations, since ERβ homodimers antagonized by tamoxifen would be unlikely to be able to compete with activated ERα. Taken together these results indicate that ERα/ERβ heterodimeric complexes can utilize the full capacity of the ERα transactivation function but only when ERβ is fully active. Strikingly, ERα does not reciprocally inhibit ERβ activity under reverse conditions, as our results with genistein demonstrate. Instead, transcriptional activity in response to genistein is enhanced when ERα and ERβ are co-expressed to levels which could not be obtained with either receptor alone. This intriguing observation suggests that co-localization of ERα and ERβ may affect estrogen signaling properties in a manner that cannot be predicted solely from studies of ER homodimers.

The Gal4 chimeric proteins which lack the AF-1 domains demonstrate similar characteristics in the responses to different concentrations of E_2, tamoxifen and genistein as do the full length receptors, suggesting

that selectivity in transcriptional activity induced by different agonists and antagonists are determined by inherent differences between ERα and ERβ in the respective LBDs. Furthermore, we observed only minor differences in transcriptional activity between the individual ERα or ERβ LBD when fused to the Gal4 DBD, in sharp contrast to our observations with full length receptors where the activity of ERβ was merely 40% compared to ERα. This observation indicates that the differences in activity between ERα and ERβ in this cellular context largely depend on the N-terminal AF-1 domains, where the ERα AF-1 makes a significantly higher contribution to the transcriptional activity of ERα than ERβ AF-1 does to the activity of ERβ. Co-expression of Gal4-αDEF and Gal4-βDEF at conditions unfavorable to one or the other ER subtype resulted in intermediary reporter activity which is in agreement with observations by Tremblay *et al.* (1999) that α/β heterodimeric complexes were transcriptionally active when only one subunit was able to bind ligand although to a lesser degree than with both subunits ligand-bound.

Taken together these results suggest that the magnitude of the transcriptional response by ERα homodimers as well as putative α/β heterodimeric complexes in this cellular background, is significantly influenced by the N-terminal part of in particular ERα, whose AF-1 to a high degree contributes to the transcriptional response. The AF-1 of ERβ, on the other hand, appears to have only a minor effect on the transcriptional activity, which is in agreement with previous observations by others. Furthermore, part of the resistance to tamoxifen antagonism demonstrated by wild type ERα, seems to be due to the agonistic effect which is primarily mediated via the ERα AF-1, as 1 μM tamoxifen antagonized the E_2-induced activity of Gal4-αDEF more strongly compared to full length ERα. The activity of ERα AF-1 has been demonstrated to act in synergy with the liganded AF-2 through a physical interaction. Therefore the mechanism by which ERβ represses ERα activity could involve modulatory effects on the configuration of the AF-2/AF-1 interaction. At E_2 concentrations where ERβ AF-2 is not activated or in the presence of tamoxifen, the ERα AF-1 may be repressed by the inactive ERβ AF-2. In the presence of genistein at concentrations which preferentially activates ERβ, activity of the strong ERα AF-1 is supported, thus explaining the high transcriptional response in the presence of both ERα and ERβ compared to either receptor alone. This would imply intermolecular contacts between the AF-1 of one subunit and AF-2 of the second subunit taking place within the heterodimeric complex, a notion which needs to be experimentally confirmed. This hypothesis is however further supported by the observation that co-expression of the Gal4-fusion proteins lacking the AF-1, only gave an intermediate response to 20 nM genistein. The N-terminus of ERα has been shown to interact with the co-activator GRIP1 in addition to the classical interaction via the AF-2 (Webb *et al*., 1998). It is possible that ERβ may interfere with this interaction or with binding to other obligate factors, or alternatively inhibit release of repressors. Differences in the populations of co-factors may contribute to cell type specific differences in ER activity and may explain why other studies carried out in different cell systems have generated other results.

We conclude that in certain cellular backgrounds ERβ has the ability to dominantly regulate the activity of the α/β heterodimer both positively (genistein) and negatively (low concentrations of E_2 and tamoxifen), perhaps through modification of the activity of ERα AF-1. Furthermore, the concentration-dependent responses to both agonists and antagonists but not the magnitude of transcriptional activity, displayed by the ERs, are largely determined by regions located C-terminally of the DBDs and cannot be explained solely by differences in the N-terminal transactivation domains. Our observations indicate that tissue selective responses to estrogen agonists and antagonists may depend on the expression and relative levels of each ER subtype.

It should be noted that all experiments described in here have been performed with the 485 aa so called short form of ERβ (lacking an N-terminal extension). In view of our observations that the responses to E_2, genistein and tamoxifen are essentially determined by the respective DEF domains, we predict that in this cell system the longer forms of ERβ would be interchangeable with the short form.

To date, information of ERβ expression at the protein level is limited. A recent study describes co-expression of ERα and ERβ protein in mouse mammary gland at different developmental stages (Saji *et al*., 2000). ERβ protein has also been demonstrated to be expressed in rat cardiac myocytes and fibroblasts where ERα is also present (Grohe *et al*., 1997, 1998). ERβ protein expression in hypothalamic parts of the rat brain appears to cooincide with ERα expression (Li *et al*., 1997). Heterodimerization between ERs is therefore likely to occur also *in vivo*.

Estrogen levels vary in females during the menstrual cycle, pregnancy and at menopause. These fluctuations may influence the estrogenic activity in tissues containing ERβ. Tissues expressing predominantly ERβ could be expected to be resistant to low levels of E_2 with respect to regulation of ERE-containing genes. The antiestrogenic effects of tamoxifen on ERE regulated genes may also prove to be more pronounced in tissues that express ERβ, a notion which may in the future need to be considered in the endocrine treatment of breast cancer. ERβ expression may also amplify the agonistic effect of the isoflavonoid genistein in tissues that also express ERα. The intake of phytoestrogens vary geographically and the population in areas where the dietary content of genistein and related compounds is high, also appears to have a lower incidence of breast cancer (Kurzer and Xu, 1997). High dietary content of phytoestrogens has therefore been proposed to be a protective factor against the development of breast tumors (Ingram *et al*., 1997). Whether ERβ, which shows a higher affinity for several phytoestrogenic compounds (Kuiper *et al*., 1997), participates in the putative cancer preventive actions of phytoestrogens remains to be determined. The concentrations of genistein required for activation of ERs and in particular ERβ are well within the range of what can be measured in the circulation of individuals on a diet rich in isoflavonoids. The presence of ERβ protein and mRNA in normal human breast tissue as well as in tumor samples suggests a putative role for ERβ in the development of breast cancer (Dotzlaw *et al*., 1999; Leygue *et al*., 1998; Sasano *et al*., 1999; Speirs *et al*.,

1999). Measurement of ERα has long been used as a diagnostic tool in the clinical evaluation of breast tumors. Clearly, assessment of the role of ERβ as a prognostic factor in breast cancer, and in the outcome of endocrine treatment is an important issue that needs to be investigated.

Materials and methods

Plasmid constructs

Wild type hERα and mERβ (N.B., the 485 aa so called short form of mERβ) in pSG5 vectors and the 2 × ERE-TK-Luc reporter construct have been described previously (Pettersson *et al*., 1997). Gal4-ERα-LBD was generated through cutting of MOR101 (Hillier *et al*., 1989) with restriction endonucleases *Fsp*I and *Bam*HI and insertion of the resulting fragment into pCMX-Gal4 (Perlmann *et al*., 1996) cut with *Eco*RV and *Bam*HI. For the Gal4-ERβ, pTKS-mERβ (Pettersson *et al*., 1997) was cut with *Bam*HI and the fragment inserted into the *Bam*HI site of pCMX-Gal4. The Gal4-TK-Luc reporter gene has been described before (Forman and Evans, 1995).

Cell culture and transient co-transfection assays

Human embryonic kidney 293 cells were routinely maintained in a 1 : 1 mix of DMEM (Gibco-BRL) and Ham's Nutrient mixture F12 (F12, Gibco-BRL) containing 7.5% fetal bovine serum (FBS, Gibco-BRL), 0.5% non-essential amino acids (NEA, Gibco-BRL), 1% L-glutamine (Gibco-BRL) and 1% penicillin-streptomycin (PEST, 100 U penicillin/ml and 100 μg streptomycin/ml, Gibco-BRL). 17β-estradiol, tamoxifen and genistein were purchased from Sigma. Cells were seeded in 24-well plates 18 h before transfection. Transient transfections were carried out using Lipofectin transfection reagent (Gibco-BRL) according to the manufacturer's description in a serum- and antibiotic-free mixture of 1 : 1 of F12 and phenol-red free DMEM supplemented with 0.5% NEA and 1% L-glutamine. The optimal amounts of ERα or ERβ expressing plasmids for the transfections were carefully determined and 5 – 30 ng were found to give maximal transcriptional efficiency. For each co-transfection experiment 100 ng of the 2 × ERE-TK-Luc reporter plasmid and 10 ng of pSG5-hERα or pSG5-mERβ respectively, or 10 ng of pSG5-hERα together with 5, 10 or 15 ng of pSG5-mERβ were used as indicated in the figure legends. All plasmid concentrations were equalized with the parental pSG5 vector. Five ng of a CMV-β-galactosidase expression plasmid were included as an internal control of transfection efficiency. The transfection medium was changed after 18 h to a phenol-red free mixture of F12 and DMEM supplemented with 5% dextran coated charcoal-treated FBS, 0.5% NEA, 1% L-glutamine and 1% PEST. E_2, tamoxifen, genistein or vehicle (0.1% ethanol) were added simultaneously. Cells were then incubated at 37°C for 24 h. The cells were lysed in 25 mM Tris-Ac, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM DTT. Luciferase activity was determined using the GenGlow system (Bio Orbit). The β-galactosidase internal control was assayed with the Galacto-Light luminescence kit (Perkin Elmer).

For the studies of chimeric receptors, 75 ng of the Gal4-TK-Luc reporter construct were used together with 10 ng of Gal4-αDEF or Gal4-βDEF, alone or in combination. The transfections, hormone treatment and analysis were carried out as described above.

Data are presented as fold induction, or as per cent of fold induction of luciferase activity corrected for the internal standard as indicated in figures and in each case represents the mean ± s.d. of at least three independent experiments performed in duplicates. Luciferase activity obtained with cells transfected with the luciferase reporter plasmid, relevant for each experiment (2 × ERE-TK-Luc or Gal4-TK-Luc), together with the parental empty expression vectors, was arbitrarily set to one.

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