Mutations in the Estrogen Receptor DNA-binding Domain
Discriminate between the Classical Mechanism of Action and
Cross-talk with Stat5b and Activating Protein 1 (AP-1)*

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Estrogen receptors (ERs) efficiently potentiate the transcriptional activity of prolactin-activated Stat5b through a mechanism that involves the ER DNA-binding domain (DBD) and the hinge domain. We have identified residues within the DBD of ER that are critical for the functional interaction of ER with Stat5b. We show that disruption of the second zinc finger structure abrogated cross-talk between ER and Stat5b, while the structure of the first zinc finger was not important. Furthermore, we confirm that intact DNA binding activity was not required for potentiation of Stat5b activity and that the dimerization of ER did not seem to be involved. Ligand-bound ERs also modulated activating protein 1-dependent transcription, and our data demonstrate that both zinc finger structures of the ER DBD are important for an intact response. We show that introduction of various point mutations within the DBD altered the response of the receptor to 17β-estradiol and to the estrogen antagonists 4-hydroxytamoxifen and ICI 182,870 on the collagenase promoter. These findings provide new insights into the mechanisms by which ERs act in cross-talk with non-related transcription factors.

Estrogens are powerful mitogens that promote growth and proliferation in many target organs. Their effects are mediated by two related nuclear hormone receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). These receptors belong to a large superfamily of nuclear hormone receptors that share a well conserved DNA-binding domain (DBD) and a structurally conserved ligand-binding domain. The N-terminal domains of these receptors, on the other hand, do not resemble each other (1, 2). The classical mechanism of activation of ERs depends on ligand binding to the receptor following which the receptor dimerizes and binds to estrogen response elements (EREs) located within the promoters of estrogen-responsive genes (for review, see Ref. 3). Ligand binding also induces a conformational change in the ligand-binding domain of the receptor, which allows the recruitment of co-activator proteins (4, 5).

The ER is also able to regulate gene expression in the absence of DNA binding by modulating the activities of other transcription factors. This mechanism is referred to as cross-talk and is common for several nuclear receptors (for review, see Ref. 6). We recently showed that ERα and ERβ efficiently potentiate the transcriptional activity of Stat5b when Stat5b is bound to the β-casein promoter upon prolactin (Prl) stimulation (7). We demonstrated that ERs and ERβ can interact with Stat5 through the DBD/hinge domain, and furthermore, we showed that the interaction of ER with classical co-activator proteins is dispensable for the potentiation of Stat5 activity. Ligand-bound ERs have also been demonstrated to up- and down-regulate the transcription of genes that contain AP-1 sites, binding sites for the Jun-Fos complex, in a cell- and ER subtype-specific manner (8–11). In addition, ER enhances the transcription of genes containing SPI-binding sites (12) and, conversely, represses the transcription of NF-κB (13, 14), GATA-1 (15), and CCAAT/enhancer-binding protein (14) when these transcription factors are bound to their cognate DNA-binding sites. The mechanism by which such cross-talk controls the expression of genes is not completely understood, but it is believed that the DNA binding activity of ER is not involved. The discovery of this mechanism would explain how estrogens regulate genes in which no consensus ERE has been found.

In this study, we further examined the domain of ER required for a functional interaction with Stat5b. We show that specific residues within the second helical structure of the ER DBD are essential for the potentiation of Stat5b transcriptional activity, whereas intact DNA binding activity per se is not required. We also analyzed the influence of various ER DBD mutations on the regulation of AP-1-dependent transcription and propose that distinct parts of the ER DBD distinguish between the classical mechanism of action and cross-talk with Stat5b and AP-1.

EXPERIMENTAL PROCEDURES

Plasmids—The β-casein (−344 to −1) luciferase reporter was provided by Bernd Groner (Frankfurt, Germany) (16), the human Stat5b expression vector was provided by Julian Ng (Imperial Cancer Research Fund, London, UK), the long form of the prolactin receptor (Prl-R) was provided by Paul Kelly (Paris, France) (17), the collagenase (−73 to +63) luciferase reporter was provided by Peter Kushner (University of California, San Francisco, CA) (8), and the TRE-tk-luc reporter was provided by Sam Okret (Karolinska Institutet, Huddinge, Sweden). The following plasmids have been described previously: ERE-tk-luc (18), human ERβ expression vector pS5G-ERβ (19), and mouse ERα expression vector pMT2-MOR (20). The L206A, R207A/R208A, Y210A, K175A/K176E, E167A/G168A, C149A/C152A, A187T, and P186T mutants were constructed by site-directed mutagenesis (QuikChange®, Stratagene) with oligonucleotide primers designed to introduce specific point mutations in pS5G-ERβ. The C201A/C204A, S200A, S200E, and ERβ A122–226 mutants were constructed by PCR techniques and subsequent
subcloning into pSG5-ERβ. The DNA sequences of the constructed ERβ mutants were verified by sequencing.

Cell Culture and Transient Transfection Techniques—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HC11 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 5 μg/ml epidermal growth factor (human recombinant, Sigma), and insulin (Actrapid, Novo Nordisk, Bagvaerd, Denmark). For transient transfection assays, COS-7 and HC11 cells were seeded in DMEM free of phenol red (Invitrogen) supplemented with 5% dextran-charcoal-stripped FBS (HyClone Laboratories, Inc.) in 24-well microtiter plates 24 h before transfection. Cells were transfected with 1 μg of reporter plasmid and 250 ng of pCMV-βGal plasmid as an internal control and various expression plasmids, as indicated in the figure legends, together with an empty expression vector to give a total amount of 2 μg of DNA/well using a modified calcium phosphate coprecipitation method (21). The transfection medium was changed after 24 h to DMEM free of phenol red. The hormones 17β-estradiol (E2) (10^{-8} M) (Sigma), 4-hydroxytamoxifen (OHT) (10^{-7} M) (Sigma), ICI 182,780 (10^{-7} M) (Torris Cookson, Inc.), and ovine Prl (5 μg/ml) (Sigma) were added as described in the figure legends. After 24 h, the cells were harvested, and luciferase was measured as described previously (7).

Expression of the various ERβ proteins was confirmed by Western blotting using an ERβ antibody (Upstate Biotechnology, Inc.).

RESULTS AND DISCUSSION

Mutations within the Second Helical Structure of ERβ DBD Disrupt Functional Interaction with Stat5b—We have previously reported that ER-mediated potentiation of Stat5b transcriptional activity through cross-talk in the nucleus requires the DBD/hinge domain (7). To determine which amino acids within this domain are important for this potentiation, we have introduced point mutations into the DBD region of ERβ. The mutant receptors were analyzed for their functional activity on a classical ERE reporter, and they were analyzed in cross-talk with Stat5b on the Stat5-responsive β-casein reporter by transient transfection assays in COS-7 cells. The ERE reporter includes two consensus ER DNA-binding sites (18), and the β-casein reporter comprises a fragment of the β-casein promoter (−344 to −1) and includes two Stat5-binding sites (16). The structure of the ERβ DBD and the location of the mutations that we introduced are shown in Fig. 1A. Data in Fig. 1C confirm the results we have reported previously (7). The wild-type ERβ efficiently potentiated transcription from the β-casein reporter, the transcription being increased in the presence of Prl by a factor of 5 over the level seen when Stat5b was present alone. The transcription was increased even further in the presence of Prl plus E2. Interestingly, mutations introduced within the amphipathic helix beyond the second zinc binding motif (L206A, R207A/K208A, and Y210A) completely eliminated the ability of ERβ to potentiate the activity of Prl-activated Stat5b (Fig. 1C), whereas the transcriptional activity on the classical ERE reporter was retained (Fig. 1B). These results show that this region of the ER DBD has an important function in cross-talk with Stat5b. Conversely, mutation of residues within the “P-box” of the recognition helix (K174A/R175E and E167A/G168A), which is known to be involved in direct interaction with DNA (22), resulted in the loss of activation of the ERE reporter (Fig. 1B), whereas the ability of the receptor to potentiate transcription from the β-casein reporter remained intact (Fig. 1C). These results demonstrate that the DNA binding activity of ER is not required for functional interaction with Stat5b. Also shown in Fig. 1C, and as reported previously (7), a DNA binding-defective mutant in which two cysteines in the second zinc finger (C201A/C204A) had been changed to alanines was unable to act in cross-talk with Stat5b, suggesting that an intact structural conformation of the DBD is required. However, a corresponding disruption of the first zinc finger (C149A/C152A) did not interfere with the ability of ERβ to potentiate Stat5b activity, defining the region required for functional interaction with Stat5b to the second zinc binding motif of the DBD and the following amphipathic helix. As expected, neither of the ERβ zinc finger mutants were able to activate the ERE reporter (Fig. 1B). We also analyzed the importance of residues known to participate in the dimer interface (22). Whereas substitution of serine 200 for alanine (S200A) did not affect the ability of the receptor on the ERE-

![Fig. 1. Point mutations within the second zinc finger of the ERβ DBD eliminate potentiation of Stat5b activity.](http://www.jbc.org/)

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driven reporter, substitution for glutamic acid (S200E) resulted in minimal activation of the reporter (Fig. 1B). Mutation of the corresponding serine residue within ERα to glutamic acid (S236A) prevents DNA binding by inhibiting the dimerization of ERα in the absence of ligand (23). The fact that the S200E mutant was still able to potentiate the transcriptional activity of Stat5b in response to Prl alone (Fig. 1C) indicates that potentiation of Stat5b activity is a function of an ER monomer. Likewise, substitution of alanine 187, which lies within the “D-box” of the second zinc finger, for threonine (A187T) also resulted in diminished transcriptional activity on the ERE reporter (Fig. 1B), although the mutated receptor was still able to potentiate transcription from the β-casein reporter (Fig. 1C). We also analyzed whether the proline residue adjacent to alanine 187 is important for dimerization since the corresponding position within the glucocorticoid receptor DBD is crucial for dimerization (24). However, substitution of this proline for threonine (P186T) did not affect the activity of the receptor on the ERE-driven reporter (Fig. 1B), indicating that mutation of this residue does not inhibit dimerization of ER. This substitution also did not affect cross-talk with Stat5b (Fig. 1C). As expected, deletion of the entire DBD (∆122–226) completely eliminated potentiation of Stat5b activity (Fig. 1C) as well as activation of the ERE reporter (Fig. 1B), confirming that an intact domain is required for functional interaction with Stat5b. Notably, similar results to those described above were obtained in transient transfection assays using the mouse mammary epithelial cell line, HC11 cells (data not shown), confirming that the observed phenotypes of the ERβ DBD mutants are not restricted to a certain cell type. Western blot analyses of cell extracts confirmed equal expression of the wild-type and mutant receptors (Fig. 1D).

Both Zinc Finger Structures within the ERβ DBD Contribute to Transrepression of AP-1.—The finding that the introduction of specific point mutations into the ERβ DBD abrogated potentiation of Stat5b transcriptional activity prompted us to analyze whether this was a common phenomenon in cross-talk between ER and non-related transcription factors. We examined the ability of ERα and ERβ to modulate AP-1-dependent transcription using the coll-73-luc reporter, which comprises a fragment of the collagenase promoter (–73 to +63) and includes a single AP-1-binding site (8). In HC11 cells, the collagenase reporter was repressed 2-fold in the presence of E2, while transcription was induced 2.5-fold in the presence of the full estrogen antagonist ICI 182,870 when cells were cotransfected with ERα or ERβ expression plasmids (Fig. 2A). Interestingly, whereas the responses to E2 and ICI 182,870 were similar in the presence of ERα and ERβ, only ERβ was able to activate transcription from the coll-73-luc reporter upon stimulation with the selective estrogen receptor modulator OHT (Fig. 2A), showing that the two receptor subtypes differ in their ligand preferences. Previous studies have shown that antagonist-bound ERs induce the transcriptional activity of AP-1 (8–11). Whether AP-1-regulated promoters are actively transrepressed or induced in response to E2 may depend on the composition of the AP-1 complex (25). In accordance with our results, cell-specific ERβ-mediated transrepression of the collagenase promoter in response to E2 has been demonstrated (9) and has been recently described for ERα (11).

Fig. 2B shows the activities of the various ERβ DBD mutants on the AP-1-regulated coll-73-luc reporter upon transient transfection of HC11 cells. Interestingly, cotransfection of the L206A, R207A/K208A, and Y210A mutants, which were all found to eliminate cross-talk with Stat5b (Fig. 1C), resulted in a reversed activity by E2 with a 3-fold activation of the reporter. On the other hand, treatment with estrogen antagonists OHT or ICI 182,870 did not activate transcription. The two P-box mutants (E167A/G168A and K174A/R175E), which had no effect on the ERE reporter (Fig. 1B), displayed distinct effects on the AP-1-regulated reporter. The E167A/G168A mutant behaved as the wild-type receptor, repressing AP-1 activity in response to E2 as previously shown with the corresponding ERα mutants (11). However, the K174A/R175E mutant displayed the same reversed activity in response to E2 as the L206A, R207A/K208A, and Y210A mutants displayed. The K174A/R175E also did not respond to treatment with estrogen antagonists, indicating that the region between the two zinc binding motifs has an essential function in the modulation of AP-1-dependent transcription. Furthermore, the integrities of both zinc finger structures of the DBD were essential, and disruption of either the first or the second zinc binding motif (C149A/C152A and C201A/C204A) altered the response to ER ligands on the AP-1-regulated reporter. The requirement for both zinc fingers contrasts with the situation concerning cross-talk with Stat5b, and it suggests that the functional interaction between ER and AP-1 is sensitive to conformational changes within both zinc fingers of the ER DBD. Notably, an ERα zinc finger mutant (C241A/C244A) also induced activation of the coll-73-luc reporter in response to E2 (data not shown), demonstrating that the reversed activity obtained upon disruption of the DBD is not restricted to ERβ. Interestingly, the S200E and A187T mutants both reversed the response to E2 on the coll-73-luc reporter (Fig. 2B). These results show that residues participating in the dimer interface also contribute to E2-induced transrepression of AP-1. However, activation of the reporter in response to estrogen antagonists OHT or ICI 182,870...
was retained, suggesting that these receptor mutants act at the promoter through distinct pathways. The S200A and P186T mutants, which both displayed intact activities on the ERE-driven reporter (Fig. 1B), repressed and activated the AP-1-driven reporter (Fig. 1B). These results show that introduction of specific point mutations within the DBD eliminates ERβ-dependent modulation of AP-1 activity in COS-7 cells. However, the same mutations enable the receptor to efficiently induce AP-1 activity in response to E2 in breast epithelial cells HC11 (Fig. 2B) and MCF-7 (data not shown). These results show that introduction of specific point mutations within the DBD eliminates ERβ-dependent modulation of AP-1 activity in COS-7 cells. However, the same mutations enable the receptor to efficiently induce AP-1 activity in response to E2 in breast epithelial cells HC11 (Fig. 2B) and MCF-7 (data not shown). This activity appears to be mediated through a distinct mechanism that can be blocked by estrogen antagonists. One explanation for the difference in the way that ERβ affects AP-1-dependent transcription in different cell types may be provided by subtle changes in the structure of the DBD. The ERβ DBD may be involved in the recruitment of additional cofactors/co-repressors that bind to the liganded ER, and these may be specific for a certain cell type. Any subtle changes in the structure of the DBD may have profound effects on the recognition of such cell type-specific factors. It should be noted, however, that the level of ERβ-mediated potentiation of Stat5b activity in COS-7 cells (Fig. 1C) is similar to that of ERβ-mediated potentiation of Stat5b activity in HC11 cells (data not shown).

Our results do not allow detailed descriptions of molecular mechanisms, and any such descriptions will only be speculations. We have, however, shown that cross-talk between ERβ and Stat5b and between ERβ and AP-1 depends on overlapping but not identical residues within the ER DBD as summarized in Fig. 4. We have defined a region within the second zinc finger of the DBD that is essential for the functional interaction of ERβ with Stat5b. Furthermore, we have shown that the ER-dependent modulation of AP-1 activity is sensitive to structural changes within both zinc fingers of the DBD and that
introduction of point mutations within this domain alters the responses of the receptor to ER ligands on the collagenase promoter. We have identified ERβ mutants that discriminate between classical activation on an ERE and cross-talk with Stat5b and AP-1, and our results contribute to understanding the mechanisms by which ERs act in cross-talk with non-related transcription factors.

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