Cross-talk between Stat5b and estrogen receptor-α and -β in mammary epithelial cells

L Björnström, E Kilic[1], M Norman[1], M G Parker[2] and **M Sjöberg**

Department of Cell and Molecular Biology, Karolinska Institutet, S-171 77 Stockholm, Sweden
[1]University Research Centre for Neuroendocrinology, University of Bristol, Bristol, UK
[2]Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, London, UK

(Requests for offprints should be addressed to M Sjöberg; Email: maria.sjoberg@cmb.ki.se.)

ABSTRACT

Both 17β-estradiol and prolactin play important roles in the mammary gland, raising the possibility of functional cross-talk between the two signaling pathways. Here, we demonstrate that estrogen receptor-α (ERα) and -β (ERβ) are both able to potentiate transcription from a Stat5-responsive promoter when activated by prolactin. Potentiation was observed not only in the presence of 17β-estradiol, but also in the presence of anti-estrogens such as tamoxifen and ICI 182,780. The magnitude of the response was dependent on cell-type: in the HC11 mouse mammary epithelial cell line ERβ potentiates transcription efficiently whereas ERα showed low activity. Conversely, in COS-7 cells, both estrogen receptors were active. We show that activation domains in the N-terminus (AF-1) and the C-terminus (AF-2) of the ERs are dispensable for potentiation. The effects are dependent on the presence of an intact DNA-binding/hinge domain, which we show is capable of interacting with Stat5b *in vitro* and in HC11 cell extracts. We conclude that ERα and ERβ act as coactivators for Stat5b through a mechanism which is independent of AF-1 and AF-2.

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INTRODUCTION

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 -β (ERβ). These receptors belong to a large superfamily of nuclear hormone receptors that share a well-conserved DNA-binding domain (DBD), a structurally conserved ligand-binding domain (LBD) and an N-terminal domain with no homology between the different receptors (Parker 1993, Beato *et al*. 1995, Mangelsdorf *et al*. 1995). ERα and ERβ have been shown to activate transcription by a number of distinct mechanisms. The classical mechanism depends on ligand binding, subsequent to which the receptor dimerizes and binds to estrogen response elements (EREs) located within the promoters of estrogen-responsive genes. Ligand binding also induces a conformational change in the LBD of the receptor to allow recruitment of coactivator proteins (Bevan & Parker 1999, Glass & Rosenfeld 2000). Alternatively, phosphorylation of specific serine residues in the N-terminus, mainly through the MAP kinase (MAPK) signal transduction pathway, may result in ligand-independent transcriptional activation on DNA (Kato *et al*. 1995, Bunone *et al*. 1996, Tremblay *et al*. 1997, Joel *et al*. 1998*a*,*b*). However, it is not clear whether receptor activation upon phosphorylation takes place in the absence or presence of hormone *in vivo*.

The ER is also able to regulate gene expression in the absence of DNA binding by modulating the activity of other transcription factors. This mechanism is referred to as cross-talk, and is common for several nuclear receptors (for review see Gottlicher *et al*. 1998). For example, ERs have been demonstrated to upregulate the transcription of genes that contain AP-1 sites, binding sites for the Jun/Fos complex (Webb *et al*. 1995) and SP1-binding sites (Porter *et al*. 1997). Conversely, ER represses transcription of nuclear factor kappa B (Ray *et al*. 1994, Stein & Yang 1995), GATA-1 (Blobel *et al*. 1995) and CCAAT/enhancer-binding protein (Stein & Yang 1995) when these transcription factors are bound to their cognate DNA-binding sites. The mechanism for such cross-talk is

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not well understood but it is believed that the DNA-binding activity of ER is not involved and therefore this mechanism may provide an explanation for how estrogens might regulate genes in which no consensus ERE has been found.

Mice with no functional ERα gene are infertile, show abnormal development of the reproductive tract and fail to develop mammary glands beyond puberty (Lubahn *et al*. 1993). ERβ-deficient mice on the other hand are fertile but show a reduced number of ovulated follicles, resulting in fewer and smaller litters compared with wild-type mice (Krege *et al*. 1998). A function for ERβ during mammary gland development has not been reported but in a recent publication it was shown that both ERα and ERβ are expressed in mammary epithelial cells during lactation (Saji *et al*. 2000). Mice lacking the prolactin receptor (Prl-R) gene also fail to fully develop the mammary gland and fail to lactate (Ormandy *et al*. 1997). Prolactin binds to the Prl-R, a membrane-spanning receptor that is associated with a tyrosine kinase, JAK2 in the cytoplasm. JAK2 phosphorylates a tyrosine in the inactive, cytoplasmic form of the transcription factor Stat5, a member of the Stat family (signal transducers and activators of transcription) (Darnell 1997). As a result Stat5 dimerizes and translocates to the nucleus where it binds to DNA and regulates expression of milk protein genes (Groner & Gouilleux 1995). There are two Stat5 genes, which encode proteins that are approximately 95% identical in amino acid sequence (Liu *et al*. 1995). Milk protein production in Stat5a and Stat5b knock-out mice is either reduced or even intact (in the case of β-casein), suggesting redundancy between the two Stat5 protein isoforms (Teglund *et al*. 1998). Deletion of both Stat5a and Stat5b genes results in infertility so that it has not been possible to analyze mammary gland development in these animals (Teglund *et al*. 1998).

Due to the importance of both prolactin and estrogen during mammary gland development, and also because both hormones are thought to be involved in the development of breast cancer, we decided to investigate the possibility of a direct interaction between the transcription factors, ERα/β and Stat5. A functional interaction between the glucocorticoid receptor (GR) and Stat5 has been described on the β-casein promoter, where GR acted as a coactivator for Stat5 in response to prolactin and dexamethasone (Stoecklin *et al*. 1996).

Here, we show that the ER can act as a coactivator for Stat5b on the β-casein promoter. Interestingly, we find that ERβ is a more potent coactivator than ERα in mammary epithelial cells, but both are active in COS-7 cells. We demonstrate that ERα and ERβ are capable of interacting with Stat5 via the DBD/hinge domain but interestingly, the integrity of the activation function (AF)-2 coactivator surface on the ER is not essential. Finally, we find that estrogen antagonists are also potent stimulators of cross-talk between ERs and Stat5.

EXPERIMENTAL PROCEDURES

Plasmids

The β-casein (−344 to −1) luciferase reporter plasmid was provided by Bernd Groner (Frankfurt, Germany) (Gouilleux *et al*. 1994), the human Stat5b expression vector by Julian Ng (Imperial Cancer Research Fund (ICRF), London, UK) and the long form of the Prl-R by Paul Kelly (Paris, France). The glutathione S-transferase (GST)-MOR 121–338 (GST-ERα aa121–338) was a kind gift of Janet Valentine (ICRF, London, UK). The pSG5-ERβ DNA-binding mutant (C201A/C204A) and the N-terminal deletion mutant (148–530) were constructed by recombinant PCR and verified by automated sequencing. The N-terminal deletion mutant was tagged with a FLAG epitope. Full-length ERβ and a series of ERβ fragments (GST-ERβ (aa1–530), GST-ERβ AF-1 (aa1–148), GST-ERβ DBD/H (aa148–260), GST-ERβ AF-2 (aa260–530)) produced by recombinant PCR were subcloned into pGEX-2TK and verified by automated sequencing. The following plasmids have been described before: the mouse ERα expression vector pMT2-MOR (Lahooti *et al*. 1994), the ERα DNA-binding mutant pMT2-MOR C241A/C244A (Lahooti *et al*. 1994), the ERα N-terminal deletion mutant pMT2-MOR 182–595 (Lahooti *et al*. 1994), the ERα C-terminal deletion mutant pMT2-MOR 1–339 (Lees *et al*. 1989), the ERα AF-2 mutant pMT2-MOR L543A/L544A (Danielian *et al*. 1992), pMT2-MOR K366A (Henttu *et al*. 1997), the human ERβ expression vector, pSG5-ERβ and the ERβ AF-2 mutant, pSG-ERβ M494A/L495A (Cowley & Parker 1999), GST fusions of regions of the mouse ERα, GST-AF-1 (Kalkhoven *et al*. 1998) and GST-AF-2 (Cavailles *et al*. 1995).

Cell culture and transient transfection experiments

HC11 cells were routinely maintained in RPMI medium containing 10% fetal bovine serum (FBS) (Gibco BRL, Paisley, Strathclyde, UK), 5 μg/ml epidermal growth factor (human recombinant; Sigma Chemical Co., Poole, Dorset, UK) and 5 μg/ml

insulin (Actrapid; Novo Nordisk, Denmark). COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) containing 10% FBS. For transient transfection assays, HC11 or COS-7 cells were plated in phenol red-free DMEM containing 5% dextran-charcoal-stripped (DCS) horse serum (Gibco BRL) in 24-well microtiter plates. Cells were transfected using a modified calcium phosphate coprecipitation method (Chen & Okayama 1987) with 1 μg reporter plasmid, 250 ng pCMV-βGal plasmid as an internal control, 50 ng Stat5b expression vector, 300 ng pMT2 or pSG5 expression plasmids together with empty expression vectors pMT2 and pSG5 to a total of 1·6 μg DNA per well. The COS-7 cells were also transfected with 10 ng Prl-R plasmid. After 24 h, the cells were washed and then maintained in phenol red-free DMEM containing 5% DCS horse serum in the presence or absence of 17β-estradiol (10^{-8} M) (Sigma), tamoxifen (10^{-7} M) (Sigma), ICI 182,780 (10^{-7} M) (Tocris Cookson Inc., MO, USA) and/or ovine prolactin (5 μg/ml) (Sigma), as described in the figure legends. Subsequently, cells were washed with PBS and harvested in lysis buffer (10 mM Tris–HCl pH 8·0, 1 mM EDTA, 150 mM NaCl and 0·65% NP-40). Extracts were assayed for luciferase and β-galactosidase activity in a microplate luminometer/photometer reader (Lucy-1; Anthos, Salzburg, Austria). β-Galactosidase activity was used to correct for differences in transfection efficiency. Expression of the various ERα proteins was confirmed by Western blotting using the ERα antibodies, H-184 and MC-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Expression of the various ERβ proteins was confirmed by Western blotting using an ERβ antibody (Upstate Biotechnology Lake Placid, NY, USA) or an anti-FLAG antibody (Sigma) to detect ERβ 148–530.

DNA affinity purification and Western blotting

A biotinylated oligonucleotide (5′-AGATTTCT AGGAATTCAAATC-3′), derived from the β-casein promoter and including a Stat5 response element, was linked to streptavidin-agarose beads as described by Beadling *et al*. (1996) after annealing to an antisense strand. An oligonucleotide with two point mutations in the consensus Stat5 response element (5′-AGATTTCTATTAATTCAAATC-3′) was prepared in a similar manner. Before extraction of nuclear proteins, beads were washed and resuspended as a slurry of approximately 50% v/v beads and buffer. HC11 cells were plated in DMEM containing 20% FBS. On reaching confluence, cells were washed three times with PBS before incubation for 24 h in serum-free medium. They were then washed twice with serum-free medium before addition of hormones. Incubation was continued at 37 °C for 30 min before nuclear extracts were prepared using the method described by Dubik & Shiu (1988). Cells were collected by scraping and lysed in a solution containing 10 mM Tris–HCl (pH 7·5), 10 mM KCl, 2 mM $MgCl_2$ and 0·2% (v/v) Nonidet P-40, the cell suspension being passed eight to ten times through a 21-gauge needle. Nuclei were collected by centrifugation at 500 ***g*** for 5 min at 4 °C and extracted with 200 μl high salt buffer (20 mM Hepes (pH 7·4), 0·4 M KCl, 1 mM dithiothreitol and 20% (v/v) glycerol) over 10 min on ice. During this time the nuclei were vortexed occasionally and passed eight to ten times through a 21-gauge needle. The lysate was diluted by addition of four volumes of lysis buffer (10 mM Tris–HCl (pH 7·6), 5 mM EDTA, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 100 μM Na orthovanadate, 1% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride), then centrifuged 5 min at 10 000 ***g*** The supernatant fractions (containing a total of ∼600 μg protein) were transferred to fresh tubes for DNA affinity purification. Nuclear extracts were mixed with 10 μl beads in 1 ml lysis buffer and incubated at 4 °C for 2 h on a rotator. After washing, bound proteins were eluted in loading buffer and separated by electrophoresis through an 8% polyacrylamide gel. Proteins were transferred to Hybond-C membranes (Amersham International, Amersham, Bucks, UK) and detected by Western blotting with an ERα antibody and the Stat5 antibody, C-17 (Santa Cruz Biotechnology). Proteins were detected using the ECL chemiluminescence system (Amersham International, Amersham, Bucks, UK).

GST pull-down

HC11 cells were plated in phenol red-free DMEM containing 5% DCS horse serum. On reaching confluence, cells were washed three times with PBS before incubation for 24 h in serum-free medium. Cells were then treated with prolactin (5 μg/ml) for 30 min and whole cell extracts were prepared in buffer containing 0·4 M KCl, 20 mM Hepes (pH 7·4), 1 mM dithiothreitol and 2% glycerol, and the protein content was determined using a colorimetric method (Bio-Rad, Sundbyberg, Sweden). GST-fusion proteins were induced and purified as described earlier (Cavailles *et al*. 1995). GST or GST fusion proteins were bound to glutathione-Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) and incubated with approximately 200 μg HC11 whole cell extract in NETN buffer (0·5%

NP-40, 20 mM Tris–HCl pH 8·0, 100 mM NaCl, 1 mM EDTA, 100 μM Na orthovanadate) containing protease inhibitors. GST-ERβ full-length and GST-ERβ 260–530 were incubated with cell extract in the presence or absence of 1 μM 17β-estradiol. After a 1 h incubation, free proteins were washed away from the beads with NETN buffer. Bound proteins were eluted in loading buffer, separated on 7% SDS-PAGE and visualized by Western blotting with a polyclonal Stat5 antibody (Santa Cruz Biotechnology).

RESULTS

Potentiation of Stat5 transcriptional activity on the β-casein promoter by ERβ

To investigate cross-coupling between estrogen and prolactin signaling pathways we investigated the effects of these hormones on transcription from the β-casein promoter in HC11 cells (Gouilleux *et al*. 1994). HC11 is a clonal mouse mammary epithelial cell line that can be induced to differentiate by treatment with lactogenic hormones leading to the production of several milk proteins (Ball *et al*. 1988, Merlo *et al*. 1996). The β-casein promoter has binding sites for several nuclear factors, including two binding sites for Stat5 (Groner & Gouilleux 1995). Although HC11 cells express Stat5, there was no significant activation of the reporter after treatment with prolactin (Fig. 1A), possibly due to the presence of negative YY1-binding sites in the β-casein promoter (Meier & Groner 1994). In the presence of transiently expressed Stat5 or Stat5 plus ERα there was slight increase in transcription from the reporter when cells were treated with prolactin or prolactin plus 17β-estradiol. However, transcription was markedly increased (6-fold compared with Stat5b transfected alone) following transient expression of ERβ and further increased in the presence of prolactin and 17β-estradiol (Fig. 1A). These effects are in complete contrast to those seen when ER stimulates transcription of classical EREs. In most cell types, ERα is known to activate a reporter containing EREs more efficiently than ERβ (Tremblay *et al*. 1997, Cowley & Parker 1999) and in line with published results we found that ERα activated such a reporter two to three times more efficiently compared with ERβ in HC11 cells (data not shown). The β-casein reporter was not significantly activated by overexpression of ERα or ERβ alone, suggesting that ER acts in conjunction with Stat5. These results demonstrate that ERβ, unliganded and bound to 17β-estradiol, is able to efficiently enhance transcription of the β-casein promoter through cross-talk with transcriptionally active Stat5.

Anti-estrogens potentiate the ability of both ERα and ERβ to enhance the transcriptional activity of Stat5 on the β-casein reporter

Next, we tested the ability of anti-estrogens to block stimulation of Stat5 transcriptional activity by ERβ. Surprisingly, the partial estrogen antagonist tamoxifen enhanced the ability of both ERα and ERβ to stimulate Stat5 activation of the β-casein reporter (Fig. 1B). We also analyzed the activation of Stat5 by ERα and ERβ in response to another partial estrogen antagonist, raloxifen (not shown), and in response to the complete antagonist, ICI 182,780 (Fig. 1C), and both ligands stimulated transcription from the reporter gene. ERα- and ERβ-mediated activation of a classical ERE reporter gene requires agonist-dependent binding of co-activators to the ER, and activation is blocked by an antagonist-bound receptor. Therefore, we conclude from these experiments that the mechanism by which ERα and ERβ enhance Stat5 activation of the β-casein reporter is mechanistically different from the activation of a classical ERE reporter.

The relative potency of Stat5 activation by ERα and ERβ is cell-type specific

Next, we wished to determine whether cross-coupling between ERs and Stat5 was restricted to certain cell types. We therefore repeated the experiments in COS-7 cells, which are devoid of Prl-Rs. The cells were transiently transfected with Prl-R with and without Stat5 proteins. In the presence of transiently transfected Prl-R alone we were unable to detect any activation of the β-casein reporter, but in the presence of transiently expressed Stat5 the reporter was activated upon addition of prolactin. Both ERα and ERβ markedly stimulated transcription from the reporter gene, by 11-fold and 5·5-fold respectively compared with Stat5b alone in the presence of prolactin (Fig. 2).

The DBD but not AF-1 or AF-2 is required to potentiate Stat5

In order to identify domains in ER required for mediating the potent stimulation of Stat5 transcriptional activity on the β-casein promoter, we analyzed a number of mutant versions (see Figs 3A and 4A) for their potential to coactivate Stat5 in HC11 cells. The activities of mutant versions of ERα and ERβ were tested in the presence of prolactin and prolactin plus 17β-estradiol (Figs 3B and 4B). Results obtained with the ERα mutants are also shown in the presence of prolactin plus tamoxifen since ERα was only found to significantly activate Stat5b in the presence of tamoxifen in these

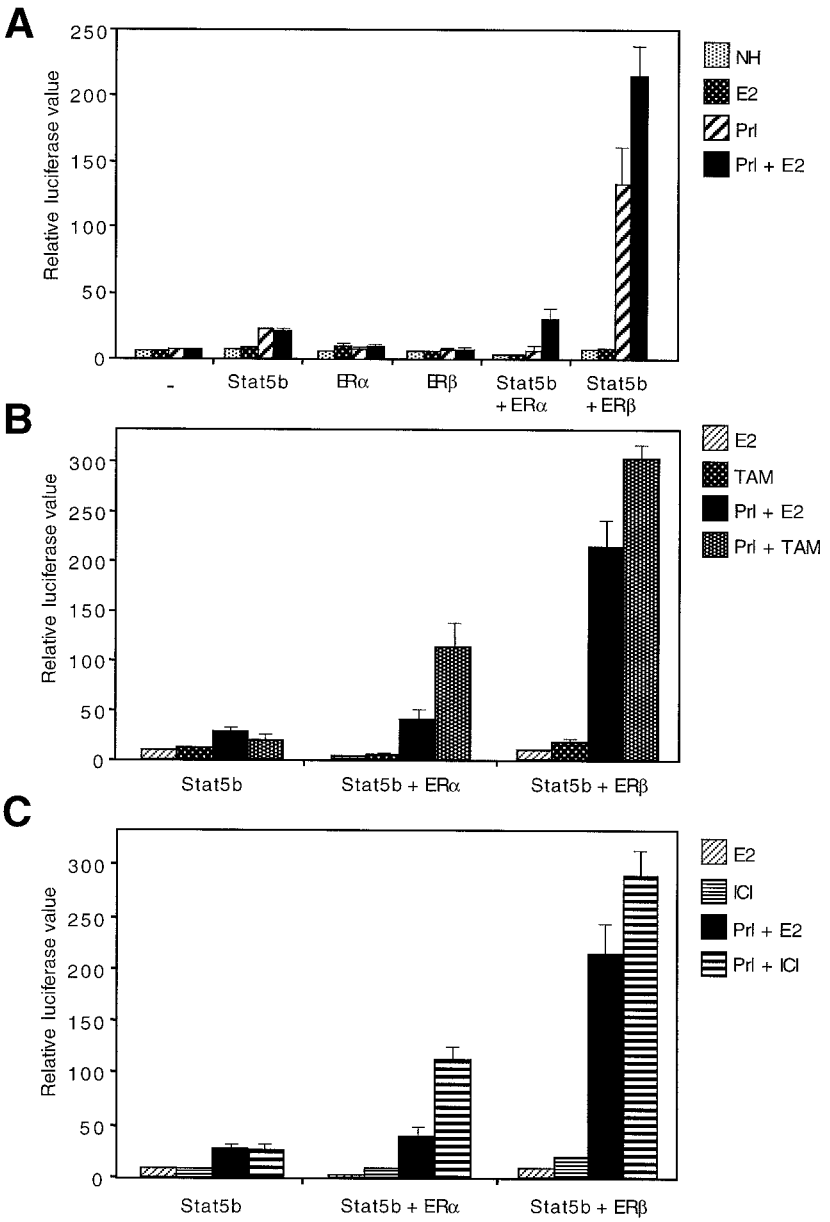

FIGURE 1. ERα and ERβ potentiate the transcriptional activity of Stat5b on the β-casein promoter. HC11 cells were transiently co-transfected with expression vectors for ERα or ERβ, Stat5, the β-casein-luciferase reporter gene and an expression plasmid encoding β-galactosidase to monitor transfection efficiency. Cells were treated as indicated, and harvested 24 h later for measurement of luciferase and β-galactosidase activity. Normalized values are shown and represent the average of a minimum of three independent experiments assayed in duplicate (means ± S.E.). (A) ERβ potentiates Stat5b transcriptional activity both in the absence of ligand and in the presence of 17β-estradiol. Cells were treated with no hormone (NH), 10^{-8} M 17β-estradiol (E2), 5 μg/ml prolactin (Prl) or E2+Prl. (B) Tamoxifen enhances potentiation of Stat5b transcriptional activity by ERα and ERβ. Cells were treated with 10^{-8} M E2, 10^{-7} M tamoxifen (TAM), Prl+E2 or Prl+TAM. (C) ICI 182,780 (ICI), a 'pure' ER antagonist also enhances potentiation of Stat5b transcriptional activity by ERα and ERβ. Cells were treated with 10^{-8} M E2, 10^{-7} M ICI, Prl+E2 or Prl+ICI.

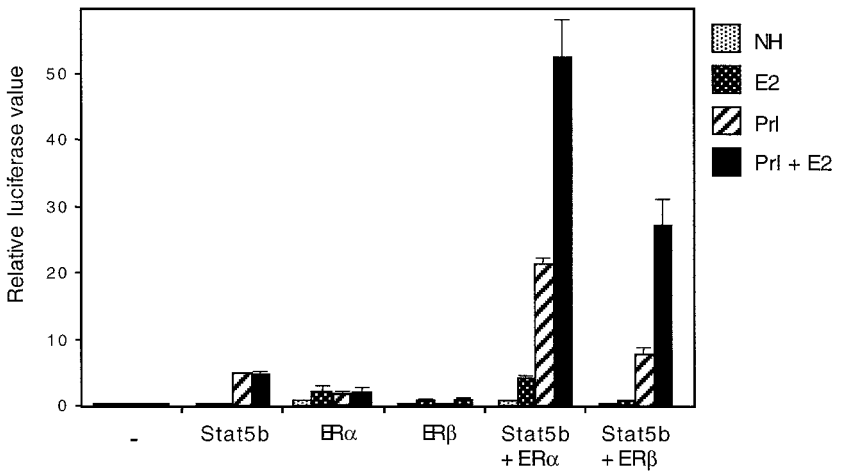

FIGURE 2. The activity of ERα and ERβ is cell-specific. COS-7 cells were transiently cotransfected with expression vectors for ERα or ERβ, Stat5b, the Prl-R, the β-casein-luciferase reporter gene and an expression plasmid encoding β-galactosidase to monitor transfection efficiency. Cells were treated with no hormone (NH), 10^{-8} M 17β-estradiol (E2), 5 μg/ml prolactin (Prl) or E2+Prl, as indicated, and harvested 24 h later for measurement of luciferase and β-galactosidase activity. Normalized values are shown and represent the average of a minimum of three independent experiments assayed in duplicate (means ± S.E.).

cells (Fig. 3B). The observation that ERα and ERβ were able to coactivate Stat5 in the presence of antagonists (Fig. 1B and C) suggests that the mechanism of activation is independent of interactions between ER and classical coactivators of the p160 family. To test this prediction, we analyzed the ability of an AF-2 mutant, which is unable to interact with p160 coactivators (Cavailles *et al*. 1995), to potentiate Stat5 activity. This mutant receptor binds ligand with affinity similar to that of the wild-type receptor (Danielian *et al*. 1992). As shown in Fig. 3B, mutations in the AF-2 domain of ERα do not interfere with 17β-estradiol- or tamoxifen-mediated activation of Stat5. An identical result was found with a transcriptionally inactive mutant of ERβ in which the corresponding AF-2 mutations were introduced (Cowley & Parker 1999) (Fig. 4B). Another mutant (K366A), which has been shown to be transcriptionally inactive and unable to bind the coactivator SRC-1 (Henttu *et al*. 1997), was also active in this system (data not shown). These results confirm that cofactors involved in cross-talk between Stat5 and ERs are different compared with those mediating classical AF-2-dependent transcription. Next, we wished to investigate the importance of the N-terminal domain of ERα and ERβ. As shown in Figs 3B and 4B, both ERα (182–599) and ERβ (148–530) N-terminal deletion mutants retain their ability to potentiate transcriptional activity of Stat5 in the presence of tamoxifen or estradiol. Phosphorylation of several serine residues in the N terminus of both ERα and ERβ has been demonstrated, and there is evidence that some of these modifications are required for full transcriptional activity (Kato *et al*. 1995, Bunone *et al*. 1996, Tremblay *et al*. 1997, Joel *et al*. 1998*a*,*b*). The results shown in Figs 3B and 4B demonstrate that none of the identified phosphorylation sites in the N termini of either ERα or ERβ is required for potentiation of Stat5 activity. Interestingly, an ERα C-terminal deletion mutant lacking most of the C terminus, ERα (1–339), is several times more active compared with ERα wild-type receptor and constitutively promotes Stat5 activation of the β-casein reporter (Fig. 3B). The latter mutant lacks most of the LBD so is unable to bind ligand, but its DNA-binding activity remains intact (Lees *et al*. 1989). This result suggests that the activity of the ERα wild-type receptor is suppressed by the presence of the LBD and that deletion results in superactivation by ERα.

Since it appeared that neither the N-terminal AF-1 domain nor the C-terminal AF-2 domain of ERα or ERβ was necessary for potentiating the Stat5 activity we next investigated the importance of the DBD. We analyzed activation by DNA-binding mutants of ERα and ERβ in which two cysteines in the second zinc-finger had been

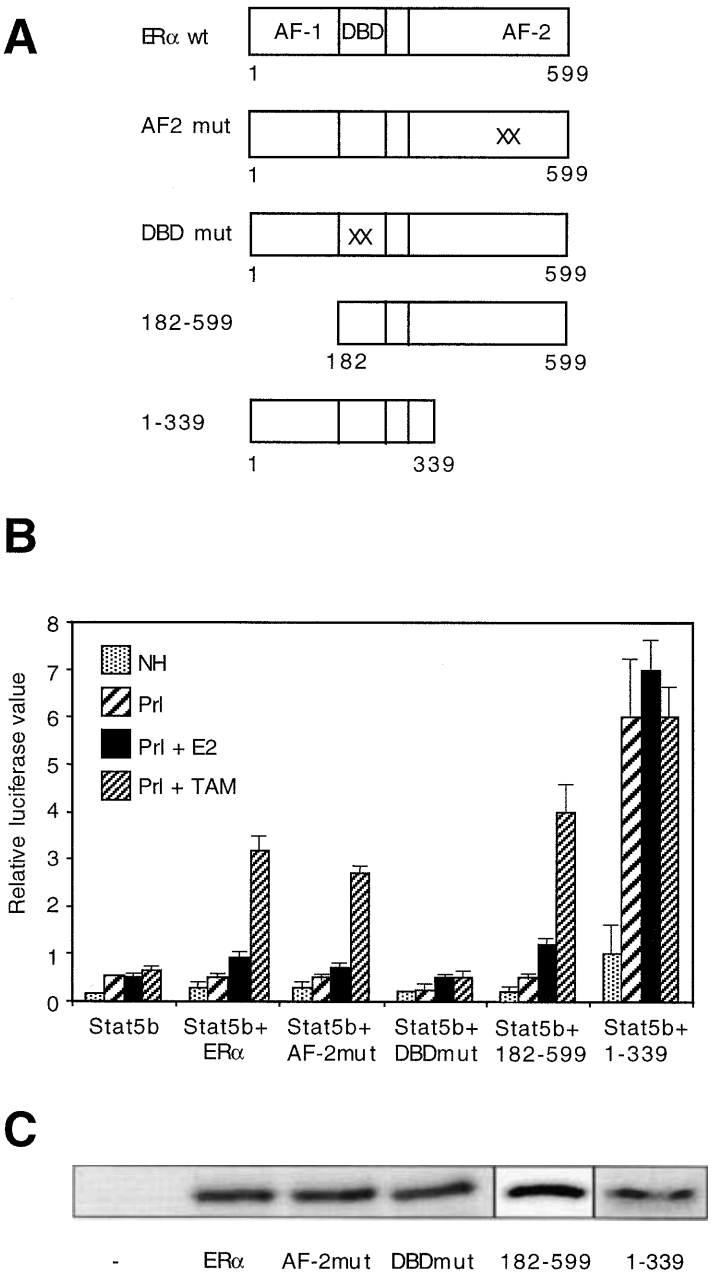

FIGURE 3. Identification of domains in ERα important for potentiation of Stat5b transcriptional activity. (A) Schematic representation of the different ERα constructs used. AF2 mut, AF-2 mutated with two point mutations in the amphipatic helix, L543A/L544A; DBD mut, C241A/C244A, defective in DNA binding; 182–599, ERα N-terminal deletion mutant; 1-339, ligand binding domain deletion mutant. (B) Activation of the β-casein-luciferase reporter gene. Cells were transfected as described in Fig. 1A. Cells were treated with no hormone (NH), 5 μg/ml prolactin (Prl), prolactin plus 10^{-8} M 17β-estradiol (Prl+E2) or prolactin plus 10^{-7} M tamoxifen (Prl+TAM). Normalized values are shown and represent the average of a minimum of three independent experiments assayed in duplicate (means ± S.E.). (C) Western blot analyses showing the expression of the various ERα proteins in transfected HC11 cells.

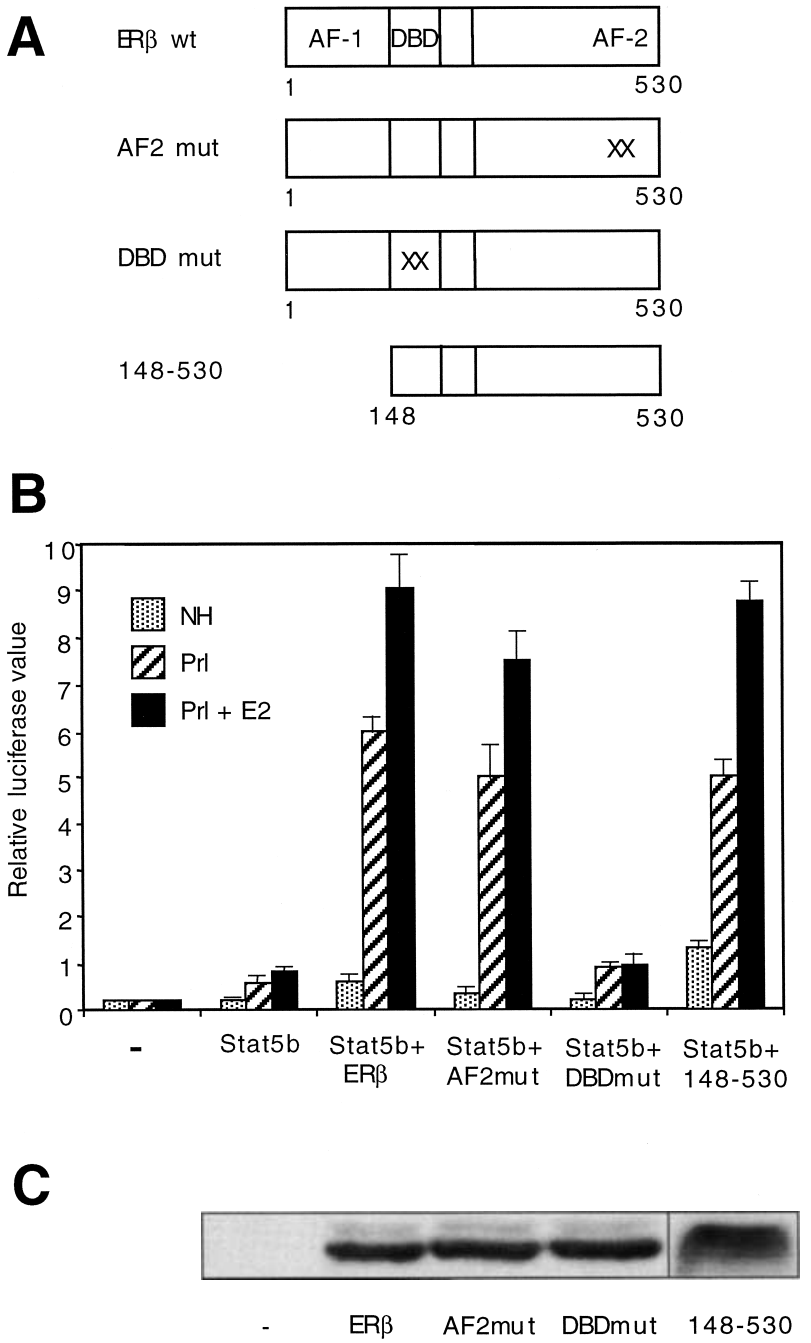

FIGURE 4. Identification of domains in ERβ important for potentiation of Stat5b transcriptional activity. (A) Schematic representation of the different ERβ constructs used. AF2 mut, AF-2 mutated, two point mutations in the amphipatic helix, M494A/L495A; DBD mut, C201A/C204A, defective in DNA binding; 148–530, ERβ N-terminal deletion mutant. (B) Activation of the β-casein-luciferase reporter gene. Cells were transfected as described in Fig. 1A. Cells were treated with no hormone (NH), 5 μg/ml prolactin (Prl) or prolactin plus 10^{-8} M 17β-estradiol (Prl+E2). Normalized values are shown and represent the average of a minimum of the three independent experiments assayed in duplicate (means ± S.E.). (C) Western blot analyses showing the expression of the various ERβ proteins in transfected HC11 cells.

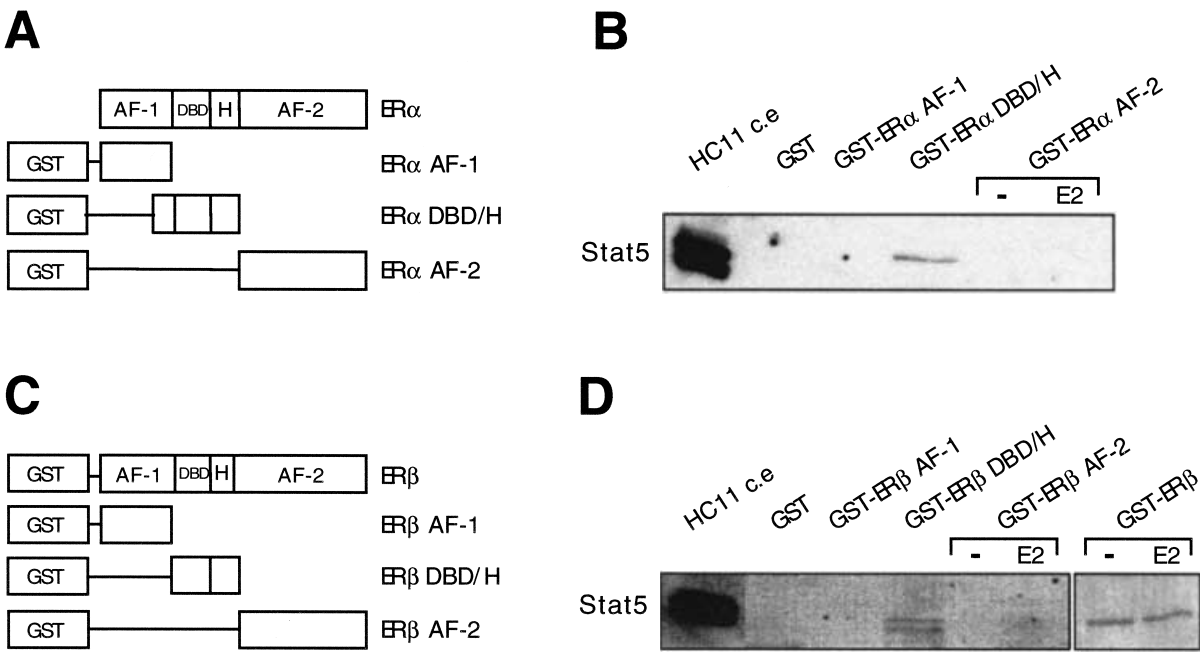

FIGURE 5. The DBD/hinge-domain of ERα and ERβ interact with Stat5b from HC11 cells *in vitro*. (A) Schematic representation of ERα and those fragments of ERα that were fused to GST and used to bring down Stat5 from HC11 cell extracts. H, hinge domain. (B) Binding of Stat5b to GST-ERα fusion proteins. GST fusion proteins were mixed with whole cell extracts prepared from HC11 cells treated for 30 min with Prl (5 μg/ml). Bound Stat5b was detected by Western blot with a Stat5b antibody. (C) Schematic representation of ERβ full-length or fragments of ERβ, that were fused to GST. Indicated regions as in (A). (D) Binding of Stat5b to GST-ERβ fusion proteins as described in (B).

changed to alanine. These mutations have previously been shown to inhibit DNA binding to an ERE (Lahooti *et al*. 1994) (result not shown). The two zinc-finger mutations abolished activation of Stat5, so an intact DNA binding seems to be required for the stimulatory effects (Figs 3B and 4B).

ERα and ERβ interact with Stat5 through their DNA-binding/hinge domain

Our observation that the DBD of ERα and ERβ is essential to promote transcription from the β-casein promoter prompted us to test whether it was capable of interacting with Stat5. GST-fusion proteins comprising truncated portions of ERα and full-length or truncated portions of ERβ (Fig. 5A and C) were incubated with cell extracts prepared from prolactin-treated HC11 cells in the presence or absence of 17β-estradiol. Bound Stat5 was analyzed by Western blot using a Stat5b antibody. The two major bands detected in HC11 cell extracts with the Stat5b antibody correspond to unphosphorylated and phosphorylated Stat5b, as demonstrated by using a Stat5b phosphotyrosine-specific antibody which only detects the more slowly migrating protein (data not shown). As shown in Fig. 5D, GST-ERβ full-length bound Stat5 both in the presence and in the absence of 17β-estradiol, whereas neither GST-ERβ AF-1 (aa1–148) nor GST-ERβ AF-2 (aa260–530) bound Stat5 under these conditions, as predicted from the transfection studies. Similarly, neither GST-ERα AF-1 (aa1–182) nor GST-ERα AF-2 (aa313–599) bound Stat5 (Fig. 5B). Both GST-ERα DBD/H (aa121–338), which contains the DBD/hinge domain and short flanking sequences of ERα (Fig. 5B), and GST-ERβ DBD/H (aa148–260), which contains the DBD and the hinge region of ERβ (Fig. 5D), bound Stat5. Thus it appears that interaction takes place through a rather small and defined domain in the receptors. Whether the interaction between ERs and Stat5 is direct or mediated via other proteins in the cell extract cannot be deduced from our data.

ERα and Stat5 associate *in vivo*

In order to analyze whether ER and Stat5 were able to interact *in vivo*, HC11 cells were used to investigate the association of ERα and ERβ with

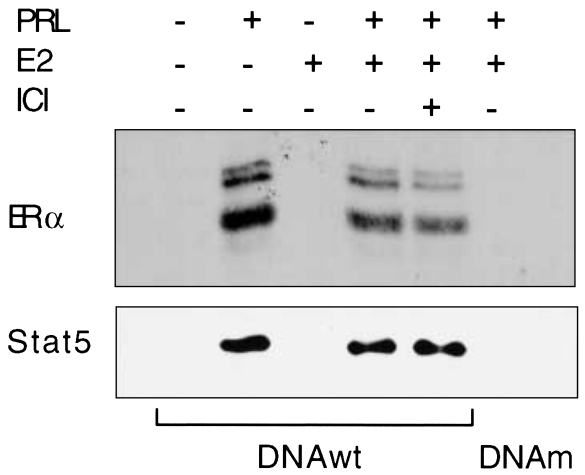

FIGURE 6. *In vivo* physical association between Stat5 and ERα in HC11 cell extract. Nuclear cell extracts prepared from HC11 cells treated for 30 min with no hormone, 5 μg/ml prolactin (PRL), 10^{-8} M 17β-estradiol (E2), prolactin plus 17β-estradiol or prolactin plus 17β-estradiol and 10^{-7} M ICI 182,780 (ICI) were mixed with a biotinylated Stat5 DNA-binding element (DNAwt) bound to streptavidin-agarose. A mutated Stat5 DNA-binding element was also included (DNAm). DNA-bound Stat5 and coprecipitated ERα were detected by Western blot using Stat5 and ERα antibodies.

Stat5 bound to a Stat5 DNA-binding element. As shown in Fig. 6, in extracts prepared from cell nuclei of prolactin-treated HC11 cells, ERα associates with Stat5 bound to a biotinylated DNA-binding site. This interaction was prolactin-dependent, demonstrating that ER requires a DNA-bound Stat5 in order to associate with the Stat5 DNA-binding element. As shown in the figure, Stat5 binds to DNA only in the presence of prolactin and it does not interact with a mutated DNA-binding element. ERα did not associate with the mutated Stat5 DNA-binding element. Furthermore, the interaction was estrogen-independent and intact in the presence of ICI 182,780, consistent with our transfection and *in vitro* interaction data shown in Fig. 1C and Fig. 5B and D. With the antibodies available we could not detect expression of ERβ in these cells.

DISCUSSION

Cross-talk between steroid hormones acting through nuclear receptors and hormones acting through other signaling pathways is likely to play an important role in many physiological responses. In this paper, we demonstrate a functional interaction between the ER and Stat5b which results in enhanced prolactin-dependent transcription in mammary epithelial cells as well as in COS-7 cells. Both ERα and ERβ appear to function as coactivators for Stat5 proteins as previously reported for GR (Stoecklin *et al*. 1996). However, the mechanism for the potentiation of Stat5 activity by the two receptors seems to be distinct. We demonstrate that the integrity of the DBD in the ER is essential whereas the N-terminal domain, but not the DBD, seems to be important in the GR interaction (Stoecklin *et al*. 1997).

Upon estrogen binding there is a conformational change in the C-terminal domain of the ER, resulting in the recruitment of nuclear receptor coactivators of the p160 family followed by activation of classical ERE-dependent transcription. In contrast, binding of antagonists does not allow such interactions (Wurtz *et al*. 1996, Brzozowski *et al*. 1997, Shiau *et al*. 1998, Pike *et al*. 1999). Potentiation of Stat5 transcriptional activity by ERβ in HC11 cells, and by both ERα and ERβ in COS-7 cells, is ligand-independent, but can be further stimulated by addition of 17β-estradiol, or, surprisingly, by ER antagonists such as tamoxifen, raloxifen and ICI 182,780 (Fig. 1B and C). These findings suggest that the interaction between ER and Stat5 allows a more flexible secondary structure of the LBD of the ER and it appears that ER-mediated ligand-dependent recruitment of coactivators of the p160 family or cAMP response element binding protein (CBP)/p300, is not required for mediating the activation of Stat5. This is further supported by our observation that AF-2 defective receptors, which are incapable of binding coactivators of the p160 family, retain the ability to potentiate Stat5 activity (Figs 3B and 4B). The N termini of ERα and ERβ have also been shown to bind p160 coactivators but this domain also appears not to be required. In addition to estrogens, both tamoxifen and raloxifen have been shown to enhance AP-1 activity on AP-1-responsive promoters (Webb *et al*. 1995, Paech *et al*. 1997), although agonist and antagonist-dependent enhancement by ER was shown to be mediated by different regions in the ER. Thus a different mechanism compared with the one described here appears to be involved (Webb *et al*. 1995, 1999).

The inability of the ERα and ERβ DNA-binding mutants to activate Stat5 suggests that the DBD zinc-finger structure is essential for proper interaction with Stat5 or, alternatively, is involved in mediating transcriptional activation by recruiting additional cofactors. The importance of the DBD is further demonstrated by the fact that *in vitro*, the

isolated DBD/hinge domain interacts with Stat5 in whole cell extracts, whereas neither the N- nor the C-terminal domain is able to do so. However, it cannot be excluded that ER binds to the β-casein promoter and thereby enhances the activity of Stat5. Although the GST-ERβ DBD/H fusion protein was able to interact with both unphosphorylated and phosphorylated Stat5b (Fig. 5D), we believe that in intact cells ER located in the nucleus will interact only with the phosphorylated protein since tyrosine phosphorylation is a prerequisite for nuclear translocation and subsequent DNA binding of Stat proteins. The importance of Stat5 DNA binding was demonstrated by the fact that a DNA-bound Stat5 was a prerequisite for ERα to be copurified on a Stat5 consensus DNA-binding element (Fig. 6).

The ERα C-terminal deletion mutant exhibited strong constitutive activation of Stat5 (Fig. 3B) demonstrating that the C terminus is not required to allow interaction with Stat5, or for mediating transcriptional activation. Furthermore, the result suggests that a suppressive activity exists in cells that is dependent on an intact ERα C terminus. Upon deletion of the C terminus the receptor may become superactive due to the release of such a suppressive activity. We propose a model whereby ERs may serve as ligands for Stat5 so that upon interaction the affinity for a cofactor (factor X) is increased. This putative cofactor may interact with ER, or alternatively with Stat5. ER ligands may modulate the affinity for such cofactors by their ability to reorganize the structure of the ER LBD (Fig. 7).

Interaction may modify the three-dimensional structure of ER and Stat5, and subsequent binding of cofactors may be affected as a result. CBP/p300 has been shown to interact with Stat5 (Pfitzner *et al*. 1998) and ER (Kraus & Kadonaga 1998), the ER interaction being mainly indirect through the p160 family of coactivators. Therefore, it is conceivable that recruitment of CBP/p300 is involved when ER potentiates the activity of Stat5. Such a mechanism seems unlikely, however, since the AF-2 protein–protein interaction surface in ER is dispensable for Stat5 activation.

Cross-coupling between Stat5 and other members of the nuclear hormone receptor family has been reported. For example it was shown that the liganded progesterone receptor enhances the transcriptional activation of Stat5 on the β-casein promoter or the c-fos promoter with, respectively, transiently expressed or endogenous progesterone receptor (Richer *et al*. 1998, Stoecklin *et al*. 1999). Transiently expressed, liganded mineralocorticoid receptor was similarly shown to enhance Stat5 transcriptional activity, whereas the overexpressed, liganded thyroid hormone receptor had the opposite effect (Stoecklin *et al*. 1999, Favre-Young *et al*.

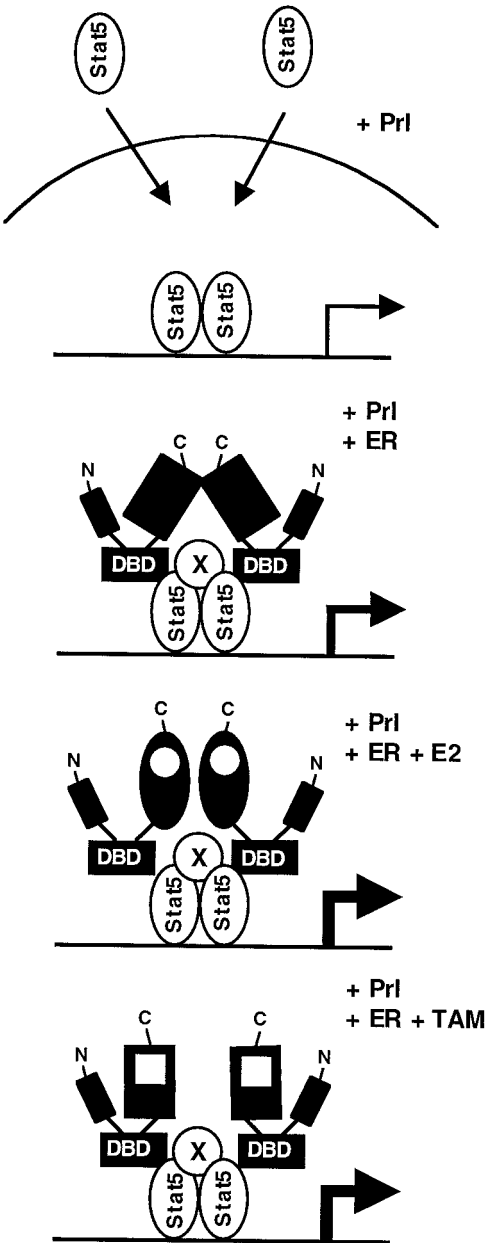

FIGURE 7. Cross-talk between ERs and Stat5. A model depicting how ERα and ERβ may act to potentiate the transcription of the β-casein promoter through interaction with Stat5, in the presence of no hormone, 17β-estradiol or anti-estrogens. Complex formation between Stat5 and ER on DNA may increase the affinity for a cofactor (factor X) binding to Stat5, or alternatively, binding to ER (not shown). (Prl, prolactin; E2, 17β-estradiol; TAM, tamoxifen.)

2000). In contrast to our results, however, overexpressed ER in the presence of estrogen was shown to decrease Stat5-mediated transcription of the β-casein promoter in COS-7 cells (Stoecklin *et al*. 1999), and ER overexpressed in COS-7 cells resulted in decreased Stat5 DNA binding and reduced Stat5 phosphorylation (Wyszomierski *et al*. 1999). Discrepancies between these studies and our results might be explained by the use of overexpressed Stat5a in the earlier studies whereas in the present study cross-talk between ER and Stat5b is analyzed.

Stat5 not only interacts with nuclear hormone receptors, but is also involved in cross-coupling with external transcribed spacers (Ets) transcription factors in the immune system (Rameil *et al*. 2000). Interestingly, GST-pull down experiments demonstrated that the Ets-1 C-terminal domain, including the DBD, was sufficient to interact with interleukin-2-activated Stat5b, *in vitro*, suggesting that interaction between DBDs of various transcription factors and Stat5 may be a common mechanism of interaction. Characterization of the Stat5 domains involved in protein–protein interactions with unrelated transcription factors will clearly be important.

We cannot rule out the possibility that intermediary proteins serve to bridge interaction between Stat5 and ER. Thus, the molecular basis for synergy between ER and Stat5 has yet to be established. It is possible, however, that since the ER is located in the nucleus in the absence of ligand (Dauvois *et al*. 1993), high levels of the receptor may be all that is required for ER-dependent potentiation of Stat5 transcriptional activity.

In conclusion, we have shown a functional interaction between Stat5 and both ERα and ERβ in mammary epithelial cells and in COS-7 cells. We have shown that the interaction takes place in the presence of estradiol as well as in the presence of estrogen antagonists, demonstrating that ERs act independently of classical coactivators. Furthermore, we have identified the region in ERα and ERβ that is functionally and physically involved in mediating the activity. The involvement of nuclear hormone receptors in various diseases makes further studies important to unravel the details of how cross-talk is regulated in order to design therapeutic reagents that might selectively block or stimulate specific pathways.

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