ARTICLE
Journal of Cellular Biochemistry 107:448–459 (2009)

Journal of Cellular Biochemistry

NF-κB and Estrogen Receptor α Interactions: Differential Function in Estrogen Receptor-Negative and -Positive Hormone-Independent Breast Cancer Cells

Nathalie Gionet,[1] Deidre Jansson,[1] Sylvie Mader,[2] and M.A. Christine Pratt[1*]

[1]*Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5*

[2]*Department of Biochemistry, University of Montreal, Montreal, Quebec, Canada H3C 3J7*

ABSTRACT

Estrogen receptor (ER)-positive breast cancer cells have low levels of constitutive NF-κB activity while ER negative (−) cells and hormone-independent cells have relatively high constitutive levels of NF-κB activity. In this study, we have examined the aspects of mutual repression between the ERα and NF-κB proteins in ER+ and ER− hormone-independent cells. Ectopic expression of the ERα reduced cell numbers in ER+ and ER− breast cancer cell lines while NF-κB-binding activity and the expression of several NF-κB-regulated proteins were reduced in ER− cells. ER overexpression in ER+/E2-independent LCC1 cells only weakly inhibited the predominant p50 NF-κB. GST-ERα fusion protein pull downs and in vivo co-immunoprecipitations of NF-κB:ERα complexes showed that the ERα interacts with p50 and p65 in vitro and in vivo. Inhibition of NF-κB increased the expression of diverse E2-regulated proteins. p50 differentially associated directly with the ER:ERE complex in LCC1 and MCF-7 cells by supershift analysis while p65 antibody reduced ERα:ERE complexes in the absence of a supershift. ChIP analysis demonstrated that NF-κB proteins are present on an endogenous ERE. Together these results demonstrate that the ER and NF-κB undergo mutual repression, which may explain, in part, why expression of the ERα in ER− cells does not confer growth signaling. Secondly, the acquisition of E2-independence in ER+ cells is associated with predominantly p50:p50 NF-κB, which may reflect alterations in the ER in these cells. Since the p50 homodimer is less sensitive to the presence of the ER, this may allow for the activation of both pathways in the same cell. J. Cell. Biochem. 107: 448–459, 2009.

KEY WORDS: ESTROGEN RECEPTOR; NF-κB; TRANSACTIVATION; REPRESSION; HORMONE INDEPENDENCE

Approximately two-thirds of breast cancers express estrogen receptor α (ERα), and its expression has been associated with a lower risk of relapse, prolonged overall survival and it is a strong predictor of responsiveness to antiestrogens such as tamoxifen [Fuqua and Cui, 2004]. The MCF-7 breast cancer cell line is an in vitro model of ER+/hormone-dependent group of cells while MDA-MB-231 and SKBr-3 cell lines represent ER-/hormone-independent cells. Clarke and colleagues have developed the MCF-7(LCC1) cell line, which are ER+ but hormone-independent by passaging MCF-7 cells in ovariectomized nude mice [Clarke et al., 1989; Brunner et al., 1993]. These cells retain the expression of the ER but are capable of growing in the absence of E2 both in vitro and in vivo. Therefore, the LCC1 subline of MCF-7 cells provides an experimental model of progression from a hormone-dependent to a hormone-independent state.

NF-κB family functions as a homo or heterodimeric transcription factor. Five members of the NF-κB/Rel family have been identified in mammals including p65 (RelA), p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelB, and cRel. All of the Rel/NF-κB family members share a highly conserved domain called the Rel Homology Domain (RHD) of about 300 amino acids, responsible for DNA binding, dimerization, association with IκB (Inhibitor of κB), and nuclear localization [Gilmore, 1999]. The canonical p65/p50 heterodimer is most abundant in ER− breast cancer cells although p50 homodimers and RelB:p52 dimers are also found in mammary tumors and cancer cell lines. NF-κB pathways have been extensively investigated in several cancers including breast cancer [Karin, 1999; Biswas et al., 2004] based on their ability to regulate genes involved in both proliferation and apoptosis. Specifically, the antiapoptotic proteins Bcl-xL and cIAP2 are both induced by NF-κB [Chu et al., 1997; Chen

Grant sponsor: Canadian Institutes for Health Research/Canadian Breast Cancer Research Alliance (CIHR).
*Correspondence to: Dr. M.A. Christine Pratt, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5. E-mail: cpratt@uottawa.ca
Received 9 September 2008; Accepted 18 February 2009 • DOI 10.1002/jcb.22141 • 2009 Wiley-Liss, Inc.
Published online 6 April 2009 in Wiley InterScience (www.interscience.wiley.com).

et al., 2000] as is the DNA damage response protein GADD45α [Zheng et al., 2005]. NF-κB also autoregulates family members including p105/p50 [Paya et al., 1992]. From the standpoint of growth, the receptor tyrosine kinase, ErbB2, is also induced by NF-κB [Kitamura et al., 2005] while the G1 cyclin, D1, is transcriptionally regulated both by NF-κB [Hinz et al., 1999] and by estrogen [Sabbah et al., 1999]. Higgins et al. [1993] have shown that proliferation and tumorigenicity of several tumor cells lines, including MCF-7 and T47D, can be inhibited by antisense oligonucleotides to RelA. Furthermore, the expression of a dominant negative form of the NF-κB inhibitor, IκB, reduced their tumorigenic potential and resistance to chemotherapeutic drugs in mouse mammary tumors [Gilmore, 1999]. Others have shown that specific inhibition of NF-κB in ER− breast cancer cell lines results in growth inhibition and cell death [Biswas et al., 2001].

An important correlation exists between NF-κB activity and estrogen dependence in breast cancer cells [Pratt et al., 2003]. Breast cancer cell lines expressing ERα contain lower levels of NF-κB activity in comparison with ER− cell lines, which display high constitutive NF-κB DNA-binding activity [Nakshatri et al., 1997; Pratt et al., 2003]. ER mutations have been observed in some cell lines selected in vitro for resistance to antiestrogens, as well as in tumor samples [McGuire et al., 1992; Wolf and Jordan, 1994]. We have shown that MCF-7/LCC1 E2-independent cells have constitutively high levels of NF-κB activity relative to the parental MCF-7 line. Others have shown that ER modulator resistance is also associated with increased NF-κB [Liu et al., 2003; Zhou et al., 2007].

Cross-talk between the ER and NF-κB has been described mainly in the context of the IL-6 promoter which is repressed as a result of an interaction between the ER and subunits of NF-κB [Stein and Yang, 1995]. Galien and Garcia [1997] found that the addition of in vitro translated ER interfered with the formation of NF-κB complexes on the IL-6 NF-κB enhancer element. RelA (p65) has been shown to be the subunit of NF-κB primarily responsible for gene activation and that interacts directly with ER [McKay and Cidlowski, 1998; Valentine et al., 2000]. Evidence also suggests that exogenous ER inhibits the ability of p50 homodimers to bind DNA [Ray et al., 1997]. A more recent study has shown that both ERα and ERβ are capable of inhibiting NF-κB-mediated IL-6 expression in a hormone-dependent manner and that repression is also dependent on dimerization of p65 to p50 [Liu et al., 2005]. ERα has also been shown to bind p65 in vitro, but this interaction has yet to be reported in vivo [Stein and Yang, 1995; Ray et al., 1997].

In previous work we showed that E2 inhibited NF-κB activity in MCF-7 breast cancer cells and that expression of a dominant negative IκBα protein prevented tumorigenicity of hormone-independent MCF-7/LCC1 xenografts [Pratt et al., 2003]. Early work by the Jordan lab and others showed that ER− MDA-MB-231 cells transfected with the ER were growth inhibited by E2 [Jiang and Jordan, 1992; Zajchowski et al., 1993; Jeng et al., 1994]. This occurred despite the induction of growth promoting factors such as TGFα in these cells. E2-independent cells have been demonstrated to become sensitive to E2 wherein the hormone acts as an apoptotic agent, rather than a growth stimulus, through an ER-mediated mechanism [Lewis et al., 2005]. E2 treatment is currently being investigated in phase II and phase III trials of tamoxifen-resistant breast tumors [Jordan, 2004].

Both the ER and NF-κB can induce expression of growth and survival genes. In the present work, we have tested the hypothesis that growth inhibition of ER− cells ectopically expressing the ERα is associated with mutual repression between NF-κB and the ER resulting in inhibition of growth and survival gene expression mediated by both pathways.

MATERIALS AND METHODS

CELL CULTURE

MCF-7, MCF-7/LCC1 breast cancer cells, and HEK293 cells were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing phenol red and supplemented with 5%, v/v, heat inactivated fetal bovine serum (FBS) (Wisent), 1% non-essential amino acids, and 10 μg/ml gentamicin (Invitrogen). Stable MCF-7 clones expressing an IκB super repressor ($I\kappa B^{SR}$) and pcDNA3 controls [Pratt et al., 2003] were maintained in high glucose DMEM supplemented as above with the addition of 2 mg/μl puromycin (Sigma). MDA-231 cells were maintained in low glucose DMEM supplemented as above. SKBr-3 breast cancer cells were maintained in the same conditions as above except that the DMEM contained 10% FBS rather than 5%. Cells were incubated at 37°C in 5% CO_2. For experiments requiring E2 depletion, cells were pre-cultured for 4–7 days in phenol-free DMEM (Invitrogen) containing 5% FBS stripped of steroids by absorption to dextran-coated charcoal. E2 (17-β-estradiol) (Sigma) was added for the indicated times at a final concentration of 10^{-8} M from a 10^{-5} M stock solution in ethanol for Western blot and electrophoretic mobility shift assay (EMSA) analysis and at a final concentration of 10^{-5} M (100 nM) for chromatin immunoprecipitation (ChIP) assays.

EMSA ANALYSIS

EMSA were performed with nuclear extracts from cultured cells as previously described [Pratt et al., 2003]. NF-κB oligonucleotides, obtained from Promega (E3291), and the canonical ERE oligonucleotide were end labeled with T4 polynucleotide kinase using [γ-^{32}P] ATP (Amersham). For supershift experiments, 2 mg of each antibody was added to extracts and left for 1 h prior to addition of the labeled probe. Equivalence of extract loading was demonstrated by EMSA with a DNA fragment containing the consensus Sp1-binding site (Promega). Samples were loaded on a 5% native polyacrylamide gel and run in non-denaturing Tris–glycine buffer.

ENUMERATION OF VIABLE CELLS

Cells were isolated by trypsinization and then stained with 0.2% trypan blue (Gibco) and enumerated using a hemocytometer. At least three samples were counted from each plate, and three different plates were analyzed for each parameter.

ADENOVIRUS INFECTION

Cells were plated at desired confluence in half of the final volume serum-free media and incubated at 37°C for 1 h. Adenovirus expressing either LacZ or the human ERα from the CMV promoter (the generous gift of Dr. S.J. Adelman [Evans et al., 2002]) was

diluted to 2.5×10^5 PFU in serum-free media and the appropriate volume was added to the cell culture depending on the desired multiplicity of infection (MOI). Following infection, the cells were incubated for 4 h after which media with serum were added. Cells were incubated for 3–4 days before harvesting.

TRANSIENT TRANSFECTIONS

MCF-7 and HEK293 cells were transfected in 60 or 100 mm dishes using the Fugene 6 Transfection Reagent (Roche Molecular Biochemicals) as per the manufacturer's protocol. Cells were seeded at $2.5–3.0 \times 10^5$ cells/60 mm plate or 7.5×10^5 cells/100 mm plate. For ERE reporter assay, cells were transfected with either a vector containing the vitellogenin ERE [Klein-Hitpass et al., 1988] driving the expression of chloramphenicol acetyltransferase (CAT) or empty vector (pcDNA3) as a negative control. For Bcl-2 luciferase assay, cells were transfected with either Bcl-2-promoter-luciferase consisting of the coding region of Bcl-2 which contains the identified ERE sequences [Perillo et al., 2000], pGL3-basic as a negative control and pGL3-control as a positive control. In some cases, cells were grown in phenol-red-free DMEM containing charcoal-treated FBS and were treated with either vehicle (0.1% EtOH) or E2 (10^{-8} M).

LUCIFERASE ASSAY

Cells were washed twice with PBS buffer and harvested in 200 μl of 1× Reporter lysis buffer (Promega). Room temperature extract was mixed with 100 μl of room temperature Luciferase Assay Reagent (Promega) and immediately placed in a Lumat LB 9507 (Berthold Technology) luminometer.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP assays were performed by using a ChIP assay kit (Upstate, 17-295) as directed by the manufacturer with some modifications. MCF-7 cells were grown to 80% confluence in phenol-red-free DMEM supplemented with 5% charcoal–dextran-stripped FBS. Cells were treated with E2 (100 nM) for 45 min after which cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. Cells were then rinsed with ice-cold PBS and collected into PBS containing protease inhibitors. Cells were then resuspended in 200 μl of lysis buffer per 1×10^6 cells, supplemented with protease inhibitors, and incubated on ice for 10 min and then sonicated. After centrifugation, supernatants were diluted 10-fold in dilution buffer and precleared at 4°C for 30 min with salmon sperm DNA-protein A-Sepharose and immunoprecipitated overnight at 4°C. Immunoprecipitation with normal rabbit immunoglobulin G (IgG) was performed to evaluate the presence of non-specific interactions, and aliquots of genomic DNA were analyzed by PCR to normalize for DNA input. Immunocomplexes were collected with 60 μl of salmon sperm DNA-protein A-Sepharose for 1 h at 4°C. Pellets were washed and eluted as per the manufacturer's directions and then incubated overnight at 65°C. DNA fragments were purified with a QIAquick Spin Kit (Qiagen). The primers used for the ChIP assays are as follows: PS2 forward primer, 5′-CAG GCC TAC AAT TTC ATT AT-3′; PS2 reverse primer, 5′-GGG GTG CCA CCG TGA CCT TCG AG-3′. For PCR, 1–5 μl from a 30 μl DNA extraction and 30–40 cycles of amplification were used.

IN VITRO TRANSLATION OF p50 AND p65

Full-length p50 and p65 were in vitro transcribed and translated using the TNT T7/T3 coupled reticulocyte lysate system (Promega) as recommended by the supplier. Radioactive translation was performed in parallel with the cold translation and the products subjected to SDS–PAGE fluorography.

GST–ER FUSION PROTEINS

GST-ER domain fusions were constructed by inserting PCR fragments from the wild-type ERα encoding the EF domain (residues 305–595), the AB domain (residues 1–181), domains C–F (residues 175–595), the C region (residues 179–264) into the *Eco*R1 site of pGEX2TK (Amersham Pharmacia Biotech) as previously described [Eng et al., 1998].

GST PURIFICATION

GST-ER domain fusion constructs were transformed into BL21 competent bacteria and production of the recombinant GST fusion protein was induced by adding 1 mM isopropylthioglycoside (IPTG) at 37°C for 4 h. Cells were recovered by centrifugation and lysed in 25 ml of 1× PBS containing 5 mM EDTA, 0.1 mg/ml PMSF, 1mg/ml lysosyme, 1% Triton-X, and one Complete Mini, EDTA-free protease inhibitor cocktail tablet (Roche Molecular Biochemicals). The lysate was sonicated six times using a Sonifier 400 (Branson) at a constant duty cycle and an output level of 2 for 30 s each time. Following centrifugation, the supernatant was incubated for 1 h on a rotating platform at 4°C with 750 ml of a 50% slurry of glutathione–sepharose 4B (Amersham) that had been equilibrated with 1× PBS containing 1 mM DTT. Beads were washed four times with 1× PBS. The purified GST fusion proteins were analyzed by SDS–PAGE.

GST PULLDOWN

One hundred microliters of glutathione–sepharose 4B bead–GST fusion protein mix was combined with 5 μl of a 35S-labeled reticulocyte lysate in a final volume of 200 μl of LSBT (20 mM HEPES–NaOH (pH 7.9), 25 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Nonidet P-40, 1% Triton X-100) containing 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated on a rotary shaker O/N at 4°C. The beads were then washed four times with LSBT and once with 50 mM Tris (pH 6.8). The bound proteins were then mixed with an equal volume of 2× SDS–PAGE gel-loading buffer. Samples were boiled for 10 min and then analyzed by SDS–PAGE.

IMMUNOPRECIPITATIONS

All IPs were performed using 1–2 mg of cell lysate mixed with antibody overnight at 4°C followed by incubation with protein A sepharose beads. After extensive washing, IPs were placed in SDS–PAGE loading buffer and process as described above.

PLASMIDS

All ERα domain proteins were generated in either pcDNA-Flag or pSG5 containing the SV40 promoter using domains as previously defined [Mader et al., 1993].

IMMUNOBLOT ANALYSIS

SDS–PAGE and immunblotting was performed as previously described [Pratt et al., 2003]. Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody, diluted to 1:5,000 in blocking solution. Proteins were detected using the Enhanced Chemiluminescence Analysis System (Amersham Biosciences) according to the manufacturer's protocol.

ANTIBODIES

The following antibodies were used for Western blot analysis: anti-ERα (HC-20) (Santa Cruz, sc-543), anti-NF-κB p50 (H-110) (Santa Cruz, sc-7178), anti-ErbB2 (C-18) (Santa Cruz, sc-284), anti-cIAP2 (H-83) (Santa Cruz, sc-7984), anti-actin (Sigma, A-2066), anti-cyclin D1 (HD-11) (Santa Cruz, sc246), anti-GADD45α (C-4) (Santa Cruz, sc-6850), anti-pS2 (FL-84) (Santa Cruz, sc-28925), and anti-Flag (M2 monoclonal, Sigma). The following secondary antibodies were used for Western blot analysis: peroxidase-conjugated AffiniPuro goat anti-rabbit IgG (Jackson Research Laboratories, Inc.); peroxidase-conjugated AffiniPuro goat anti-mouse IgG (Jackson Research Laboratories, Inc.). The following antibodies were used for supershift in EMSAs: anti-NF-κB p50 (H-119) (Santa Cruz, sc-7178), anti-NF-κB p65 (Santa Cruz), anti-ERα (Santa Cruz). Antibodies used in ChIP assays were NF-κBp50 (Upstate), NF-κB p50 (Rockland), NF-κB p65 (Santa Cruz), anti-ERα (HC-20) (Santa Cruz), and rabbit IgG (Santa Cruz, sc-2027).

RESULTS

THE EXPRESSION OF ERα IN ER-NEGATIVE AND HORMONE-INDEPENDENT BREAST CANCER CELLS DECREASES VIABLE CELLS

We first assessed the effect of wild-type ERα on cell viability in ER-negative/E2-independent and ER-positive/E2-independent cells. To this end, we infected two ER-negative breast cancer cell lines, MDA-MB-231 and SKBr-3, and the ER-positive estrogen-independent LCC1 cells with an adenovirus expressing hERα (Ad-ERα). The immunoblot in Figure 1A confirmed the level of expression of ERα in the infected cell lines at the viral MOI used for each line. After only 2 days of infection, the ERα-infected MDA-MB-231 and SKBr-3 cells began to show morphological changes (Fig. 1B). Cells expressing ERα had smaller, flattened cell bodies and began to develop processes. After 4 days of infection only 34% of ERα-infected MDA-MB-231 cells were viable relative to a LacZ control population (Fig. 1C). Similarly, after 4 days of infection with ERα only 62% of SKBr-3 cells were viable compared to the LacZ control population (Fig. 1D).

LCC1 cells are derivatives of ER-positive/E2-dependent MCF-7 breast cancer cells but display E2-independent growth dependent on constitutive NF-κB DNA-binding activity [Pratt et al., 2003]. In addition, LCC1 cells have been shown to express a high proportion of a variant ERα lacking exon 3 [Han et al., 2004]. We therefore wanted to determine what effect re-expression of wild-type ERα would have cell viability in LCC1 cells. After only 2 days following Ad-ERα infection, the cells appeared to be swollen and flat and began to take on morphological features similar to their parental MCF-7 cell line. Although LCC1 cells typically display three-dimensional growth in culture, after Ad-ERα infection these cells revert back to a more flattened spread out phenotype characteristic of less transformed cells (Fig. 1B). Four days after infection only 62% of Ad-ERα-infected cells were viable relative to a LacZ control population (Fig. 1E). These results suggest that expression or re-expression of the ERα in breast cancer cells that do not express the ER or do express it but no longer rely on the estrogen pathway for growth and survival, has negative effects on cell viability.

EXPRESSION OF ERα DECREASES NF-κB DNA-BINDING ACTIVITY IN SEVERAL BREAST CANCER CELL LINES

Based on previous studies that have reported inhibition of NF-κB by ligand bound ER and constitutive activation of NF-κB ER− cell lines, we sought to determine whether re-expression of ER in these cells lines would inhibit constitutive NF-κB DNA binding. We used an EMSA to determine the effect of expression of wild-type ERα NF-κB-binding activity in ER− and ER+ LCC1 cells. We have previously shown that MCF-7 ER+ cells have lower endogenous levels of NF-κB than LCC1 cells and that E2 withdrawal and treatment increase and decrease NF-κB activity, respectively, in MCF-7 cells. Both cells contain mostly p50 homodimers with a relatively small amount of p65:p50 dimers [Pratt et al., 2003]. Figure 2A shows that p65:p50 NF-κB levels in both cells were reduced by overexpression of the ERα with relatively little effect on the predominant p50 homodimers. NF-κB activity in ER− cells on the other hand is normally un-apposed such that introduction of the ER profoundly reduces DNA-binding activity thus producing a greater overall inhibition which is most profound for the p65:p50 complex (Fig. 2B). The relatively milder effects of ERα in LCC1 cells may be due to interactions of the transfected ERα protein with the alternatively spliced form of the ERα. Since the ER+ cells have a higher relative level of p50 homodimers to p65:p50 heterodimers, our results suggest that the heterodimer may be more sensitive to ERα expression in these cells than in p50 homodimers. Expression of the ERα strongly reduced NF-κB DNA binding in both ER− cell lines (Fig. 2B). Again this decrease was most evident in the p65:p50 heterodimer although the p50 complex was sensitive in SKBr-3 cells. In addition, in SKBr-3 cells, we observed a decrease in NF-κB binding in response to treatment with E2. Therefore, expression of ERα in breast cancer cells strongly decreases the NF-κB activity and, in some cells, this can be further reduced by the presence of E2. The effect of the ERα (and E2), however, may depend on cell-specific factors including the level of individual NF-κB complexes.

EXPRESSION OF ERα DECREASES THE EXPRESSION OF NF-κB-REGULATED PROTEINS

Although there are several reports that have shown that E2 inhibits transcription from a canonical NF-κB response element, there is a lack of information on the effects of E2 on endogenous NF-κB regulated genes. These experiments were done in ER-negative MDA-MB-231, SKBr-3, and ER-positive LCC1 breast cancer cells. Using cell extracts derived from Ad-ERα or LacZ-infected cells, Western blots were performed for either p105/p50, cIAP2, ErbB2, and/or cyclin D1, four proteins known to be transcriptionally regulated by NF-κB [Cogswell et al., 1993; Chu et al., 1997; Hinz et al., 1999;

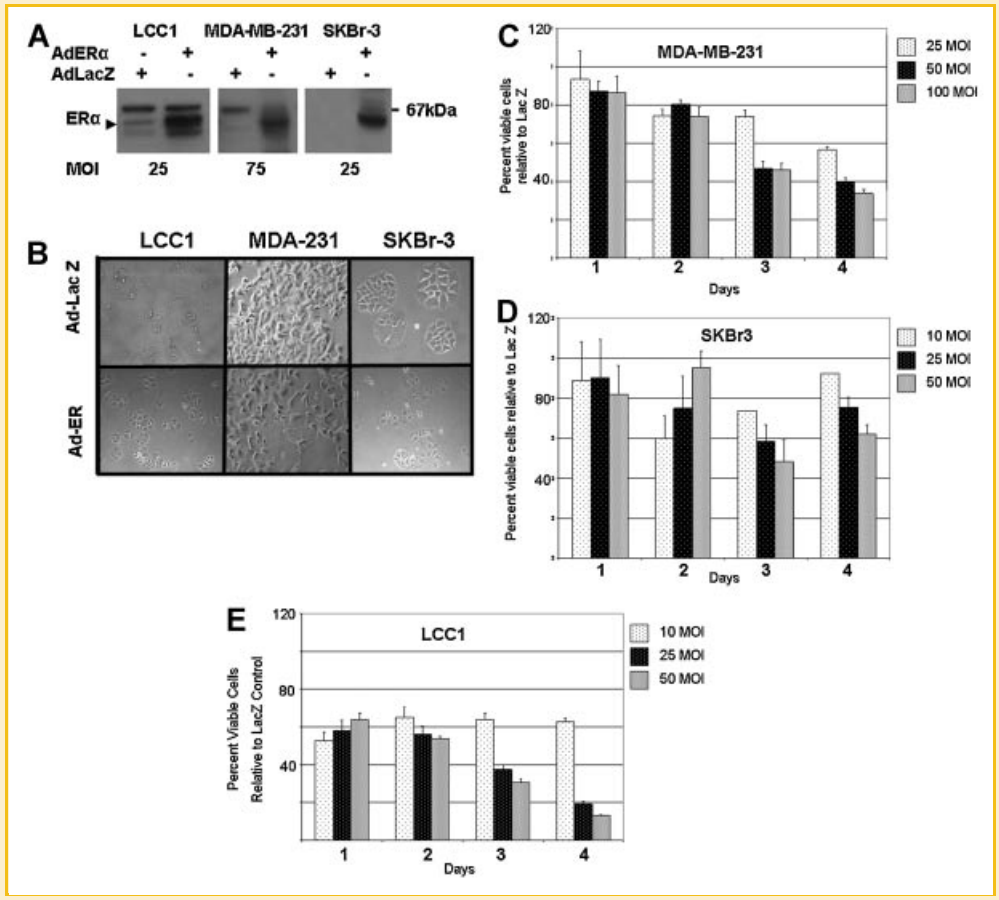

Fig. 1. Effect of ERα expression on morphology and growth. The ER-positive, estrogen-independent LCC1 cell line and two ER-negative breast cancer cell lines, MDA-MB-231 and SKBr3, were infected with Ad-ERα or LacZ. A: Immunoblot of ERα expression levels in infected cell lines at the indicated viral MOI. B: Light micrographs of Ad-infected cell lines showing changes in cellular morphology 4 days after infection. Because of differences in infection efficiencies between cell lines, different MOIs of infection were used in order to produce similar levels of ERα expression; LCC1 and SKBr3 cells (25 MOI), MDA-231 cells (75 MOI). C–E: ERα expression results in decreased cell viability. MDA-MB-231, and SKBr3 and LCC1 cells were infected with Ad-ERα or LacZ. Viable cells were counted every day for 4 days and mean values were plotted relative to LacZ control population. All experiments were done in triplicate.

Kitamura et al., 2005]. In MDA-231 cells, the expression of the ERα decreased the expression of both cIAP2 and ErbB2 (Fig. 3A) while there was only a slight decline in cyclin D1 expression. Similar results were obtained using SKBr-3 cells extracts wherein ERα expression resulted in a marked decrease in p105/p50 and ErbB2 levels. Figure 3C shows that E2 could further reduce expression of ErbB2 in SKBr-3 cells. No change was observed in cyclin D1 protein levels (Fig. 3B). In contrast to the ER-negative cells, expression of wild-type ERα in LCC1 cells had no effect on the expression of p105/p50 and only a small decrease in ErbB2 protein was observed (Fig. 3D). Again, there was only a slight decrease in cyclin D1 in LCC1 cells overexpressing the ERα, which is in keeping with the reduced effects of ERα on NF-κB activity in LCC1 cells.

IN VITRO ASSOCIATION OF ERα WITH NF-κB SUBUNITS p50 AND p65

We have shown that expression of ERα can reduce NF-κB DNA binding and that the reduced DNA binding is associated reduced NF-κB activity and lower levels of NF-κB-regulated proteins. Others have shown that the ERα is capable of interacting with NF-κB subunits p50, p65, and c-Rel in vitro [Stein and Yang, 1995; Ray et al., 1997]. The results from these studies indicate a dependence on the hormone-binding domain as well as the D domain (hinge region) for ER/NF-κB complex formation. In addition, the interaction was found to be independent of E2. While the DNA-binding domain of the ERα was found to be necessary for repression of NF-κB activity, it was not required for the protein–protein interaction. Neither of these studies dissected the interaction between the ERα and p50 nor did they examine the interaction between full-length p65 and ERα fragments. To further define the mechanism of ER inhibition of NF-κB, we generated pGEX expression vectors containing a series of ERα deletion mutants (Fig. 4A). GST-fusion proteins (Fig. 4B) were then used in a pulldown assay using in vitro transcribed and translated [35S]-Met-labeled wild-type p50 and p65 (Fig. 4C). Specific binding of GST-ER(CDEF) fusion protein to both p65 and p50 was observed (Fig. 4D). Shorter deletion mutants of ERα encompassing only the AB domains, EF domains, or C domain were unable to bind p50 although we did observe a strong interaction

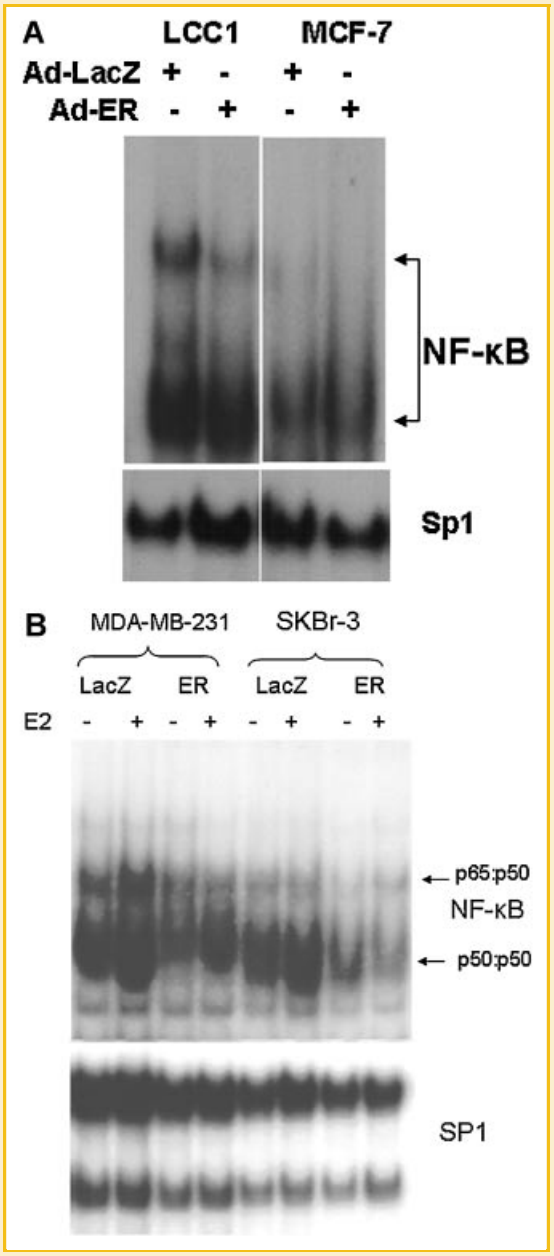

Fig. 2. ERα expression decreases NF-κB DNA binding. A: ER-positive MCF-7 and LCC1 cells were infected with adeno-ER or adeno-LacZ and subjected to EMSA analysis for NF-κB DNA binding. B: The ER-positive/estrogen-independent LCC1 cells and two ER-negative breast cancer cell lines, MDA-MB-231 and SKBr3, were infected Ad-ERα gene or Ad-LacZ. Cells were grown in PRF media for 4 days and then treated with E2 or vehicle for 4 days. Nuclear extracts were collected and subjected to EMSA using the consensus κB-response element as described in Materials and Methods Section. Arrows indicate fast and slow migrating NF-κB complexes. Five micrograms of the same nuclear extract was subjected to EMSA using the consensus Sp1 enhancer element as a loading control.

between ERα-EF and full-length p65 in the absence of the D domain. These results differ from those of Stein and Yang [1995] who observed only a weak interaction between the RHD in p65 (amino acids 1–282) and the DEF region. This suggests that the previously defined region of interaction in p65 may not confer optimal binding to the (D)EF domain. Moreover, our experiments also suggest that a broader domain of the ERα is required for p50 interaction than for p65 interaction.

To assess whether p65 and the C-terminal domains of the ERα interact within the cell, we transfected HEK293 cells with various Flag tagged ER domain constructs along with either p50 or p65 and performed co-immunoprecipitations (co-IP). The results in Figure 4E demonstrate that the ERα WT co-IPs with anti-p50 or anti-p65. A high level of expression and IP of both p50 and p65 were obtained (Fig. 4F). As expected the EF domain did not interact with p50, however, under these experimental conditions, p65 was also not associated with the EF domain. However, this may simply be a function of the lack of a nuclear localization signal within the EF region, which would preclude formation of nuclear complexes.

p50 ASSOCIATES WITH THE ER ON AN ERE IN VITRO BUT THE ER IS NOT DIRECTLY BOUND TO COMPLEXES ON THE NF-κB RESPONSE ELEMENT

Since the ER and NF-κB proteins can be demonstrated to bind in vitro, we wished to determine whether this association also takes place in DNA:protein complexes. The EMSA in Figure 5 shows MCF-7 cell nuclear extracts bound to either the ERE or the κB-response element. Extracts from MCF-7 cells produce two shifted bands on the ERE. ERα bound to ERE can be supershifted with an ERα antibody resulting in substantial loss of the unshifted complex. The p50 antibody supershifted the ERE resulting in two bands which may correspond to the two ERE complexes present in MCF-7 cells. Interestingly, the abundance of the upper band of the ERE complex did not appear to diminish although the lower band appeared reduced. Since the ERα downregulates NF-κB-mediated transcription, we next tested for the presence of the ER on the κB-response element. Interestingly, although anti-p50 clearly supershifted the NF-κB complex, the ERα antibody was unable to supershift the NF-κB complex nor did it alter the abundance of the shifted complexes. Thus, at least in the in vitro context of NF-κB:DNA complexes on an isolated κB-response element, ERα does not appear to interact directly with NF-κB complexes.

INTERACTION OF P65 WITH THE ER:ERE COMPLEX AND DIFFERENTIAL ASSOCIATION OF p50 WITH THE ER:ERE IN ESTROGEN-INDEPENDENT LCC1 CELLS

In keeping with the ability of p50 and the ERα to interact in vitro and in vivo, the data above demonstrate that p50 interacts with the ER:ERE complex in a robust manner. We next wished to determine whether there is evidence for ER:ERE:p65 complexes. Using both MCF-7 and LCC1 E2-independent ERα-positive breast cancer cells that display constitutive NF-κB activation [Pratt et al., 2003], we assessed both p50 and p65 interactions with ER:ERE complex (Fig. 6A). Supershift analysis with p65 antibody did not show a typical shift in the ERE probe; however, in both cell lines, the addition of the p65 antibody reduced the levels of the ERE probe complex. This result suggests that the interaction of the p65 antibody may have destabilized the ER:ERE complex thus releasing free probe.

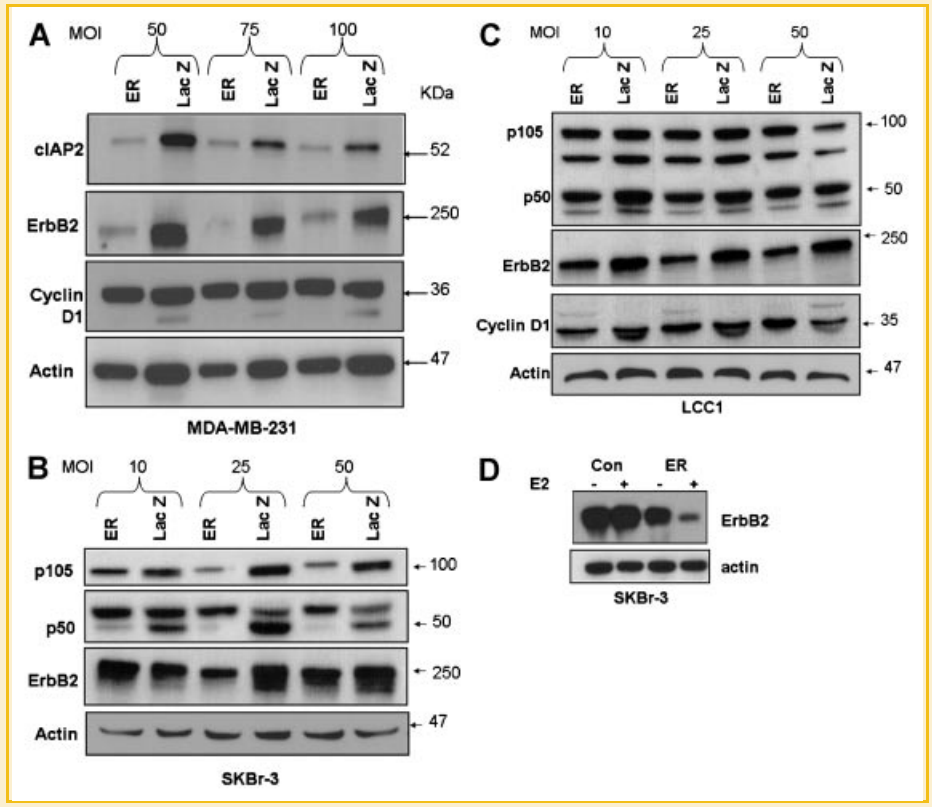

Fig. 3. ERα expression decreases expression of NF-κB-regulated proteins. ER-negative, MDA-MB-231 (A), and SKBr3 (B) cells, and the ER-positive LCC1 cells (C) were infected with Ad-ERα gene or LacZ (Z) at 75, 25, and 25 MOI, respectively. Fifteen micrograms of whole-cell protein extract was subjected to immunoblot analysis of NF-κB-regulated proteins. The expression of actin was used as an internal loading control. The numbers on the right indicate the molecular masses in kDa. D: Uninfected and Ad-ERα-infected SKBr3 cells were cultured in phenol-red-free medium and then treated with E2 or vehicle for 72 h. Lysates were immunoblotted for ErbB2.

The addition of p50 antibody to the MCF-7 ERE complex again resulted in a supershift of two discreet bands; however, in contrast to the MCF-7 complexes, only one band was apparent in the supershift. Thus, ERα interaction with p50 in at least one of the ER:ERE complexes in LCC1 cells appears to have been eliminated. A possible explanation for the differential association of ER and NF-κB protein on the ERE in LCC1 and MCF-7 cells is the presence of the Δexon3 ERα in LCC1 cells [Han et al., 2004]. The presence of this form of the receptor these LCC1 cultures is confirmed in Figure 6B.

INHIBITION OF NF-κB INCREASES THE EXPRESSION OF E2-REGULATED PROTEINS

The results above suggested that NF-κB might interfere with ER-mediated transcription. To assess this we assayed the effects of NF-κB inhibition on the expression of ER-regulated genes. Normally, NF-κB is maintained in the cytoplasm through an interaction with its inhibitor IκB. Mutation of the IκBα serine 32 and 36 prevents both phosphorylation and degradation of the inhibitor thus inhibiting NF-κB activity. To determine whether inhibition of NF-κB would promote increased expression of E2-regulated proteins, we used MCF-7 cells that were stably expressing the mutant IκBα super repressor ($IκBα^{SR}$). The cells were maintained in phenol-red-free media for at least 4 days and treated with E2 (10^{-9} M) or vehicle for 4 days after which Western blot analysis was performed in order to verify expression levels of E2-regulated genes. Stable MCF-7 clones transfected with empty pcDNA3 vector were used as a control. In order to verify inhibition of NF-κB, we blotted for GADD45α. NF-κB inhibition has been shown to enhance the stability of GADD45α mRNA resulting in increased protein levels [Zheng et al., 2005]. Stable clones expressing $IκBα^{SR}$ had increased GADD45α protein levels compared to control cells (Fig. 7A). In addition, treatment with E2 further increased the protein levels of GADD45α, which demonstrates that E2 may also be able to interfere with inhibitory NF-κB activity. In order to determine what effect NF-κB inhibition had on E2-regulated genes, we also immunoblotted for Bcl-2 and pS2. The lower panels demonstrate that expression of $IκBα^{SR}$ increased Bcl-2 and pS2 protein levels which were further elevated by treatment with E2.

To confirm that these results were a function of transcriptional repression we transiently transfected MCF-7($IκBα^{SR}$) cells with either the Bcl-2 or vitellogenin ERE–promoter–reporter constructs (Fig. 7B,C). Cells expressing the $IκBα^{SR}$ demonstrated a 1.7-fold increase in basal Bcl-2 reporter gene activity compared with control while the vitellogenin ERE activity increased by 4-fold in these cells over control cells. Note that although Bcl-2 can be regulated by both E2 and the non-canonical NF-κB proteins [Viatour et al., 2003], IκB will only have an inhibitory effect on the canonical pathway, which predominates in ER+ cells [Pratt et al., 2003].

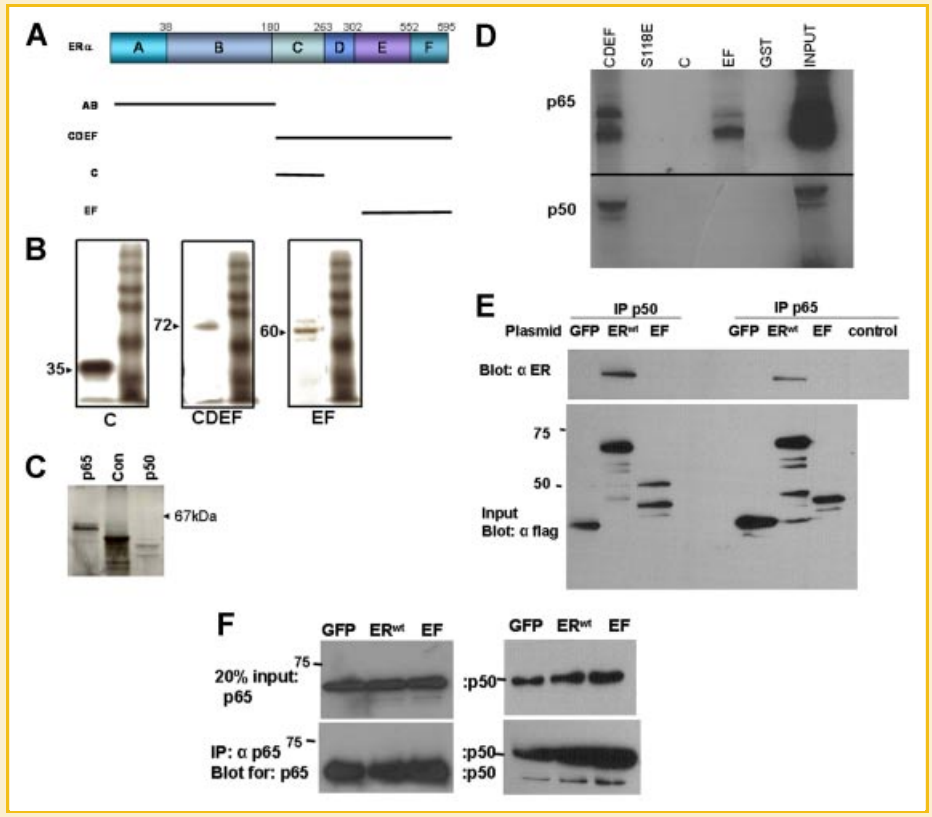

Fig. 4. ERα interacts with NF-κB in vitro. A: GST-ER contains amino acids 1–180 corresponding to the A and B. GST-ER-CDEF contains amino acids 180–595 corresponding to the C–F domains. GST-ER-C contains amino acids 180–263 corresponding to the C domain. GST-ER-EF contains amino acids 302–595 corresponding to the E and F domains. Expression vectors containing ERα deletion mutants were expressed in bacteria by use of the pGex expression vector system. B: A Coomassie blue-stained gel of the purified GST-ER proteins is shown. C: Wild-type p65 and p50 were in vitro transcribed and translated and [35S]-methionine-labeled protein subjected to electrophoresis and fluorography. The lysate in the middle lane was programmed with the control luciferase transcript. D: The GST-pulldown assay was performed in the absence of E2 as described in Materials and Methods Section. The experiment was repeated three times with similar results. Molecular weight is indicated in kDa. E: HEK293 cells were transfected with Flag-ERα^{WT}, Flag-ERα-EF, or Flag-GFP with either p65 or p50 and lysates subjected to IP using anti-p50 or anti-p65 followed by immunoblot for Flag as detailed in Materials and Methods Section. The lane marked control is a mock IP without antibody. F: Input proteins (20% of total) and p65 and p50 IP immunoblotted to detect p65 and p50, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

These results suggest that NF-κB may tonically inhibit ERE transactivation and reduction in NF-κB activity alleviates repression of the ER allowing an enhanced response to E2.

ESTROGEN RECRUITS NF-κB PROTEINS TO AN ERE IN VIVO

Although significant work has been done to investigate the inhibition of NF-κB activity by the ER, little has been done to define the role of NF-κB in the inhibition of ERα-mediated transcription. Expression of the IκBα^{SR} protein in cells demonstrated that a number of key E2-regulated proteins were markedly increased by inhibition of NF-κB. To assess whether or not NF-κB interacts with the ER on an ERE in chromatin, we examined the recruitment of ERα and NF-κB subunits p50 and p65 to the promoter of the endogenous E2-responsive gene, pS2. MCF-7 cells were grown in normal media (experiment 1) or phenol-red-free media for 4 days (experiment 2) and treated with either E2 (10^{-8} M) or vehicle for 45 min. The status of the endogenous complex present on the estrogen responsive regions was determined using ChIP. Following ChIP with either anti-ER, anti-p50, or anti-65 antibody DNA was extracted and interactions between ERα, p50, and p65 were analyzed by PCR using a specific pair of primers spanning the ERE of the pS2 promoter. As shown in Figure 8A, ERα, p50, and p65 demonstrated occupancy on the pS2 promoter even in the absence of E2. The presence of E2 further increased recruitment of the ER and NF-κB to the pS2 promoter in experiment 2, which was performed in phenol-red-free medium. Negative control primers derived from outside this region of the pS2 gene failed to amplify from the ChIP complexes. Figure 8B shows the input amplification for experiment 2 using 10-fold more input than in the full panel. Thus, in association with the ERα, both p50 and p65 can form complexes on an ERE in vivo.

DISCUSSION

Most studies of ER:NF-κB cross-talk have focused on the inhibition of NF-κB-mediated transcription by the ER. Much of this work has been done in the context of the IL-6 promoter wherein an ER mutant devoid of the DNA-binding region was unable to repress promoter activation [Ray et al., 1997], even though the DNA-binding domain

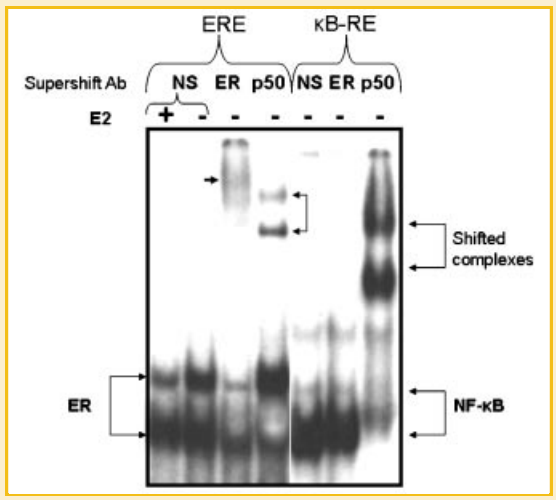

Fig. 5. p50 and ERα complexes on the ERE and the NF-κB response element in vitro. Nuclear extracts from MCF-7 cells grown in PRF medium for 4 days followed by 48 h with E2 or vehicle, were subjected to EMSA on the ERE and NF-κB consensus oligonucleotides. Supershift analysis was performed using anti-p50 and anti-ERα. Arrows indicate basal and shifted complexes.

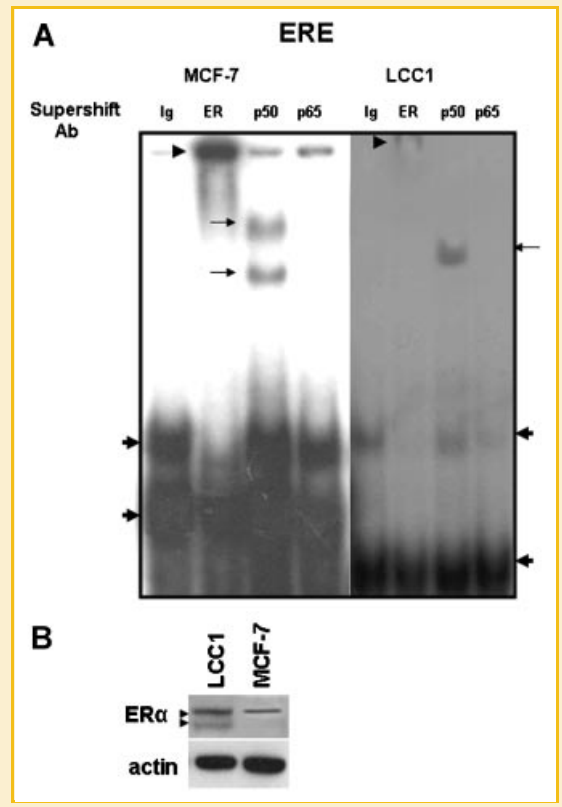

Fig. 6. Altered ERα:p50 association with the ERE in LCC1 cells. A: ER+/estrogen-independent LCC1 cells and ER+/estrogen-dependent MCF-7 cells were grown in PRF media for 4 days followed by treatment with either E2(+) or vehicle(−) for 4 days. Five micrograms of nuclear extracts was subjected to EMSA using an oligonucleotide containing the consensus estrogen response element. Complexes were supershifted with anti-p50, p65, or ERα antibody. Arrowheads indicate ER shifted complexes, long arrows, p50 shifted complexes and short arrows basal ERE complexes. B: Immunoblot of the ERα in LCC1 and MCF-7 cells. Arrowheads indicate the position of the full length and exon 3 splice variant in LCC1 cells.

is not required for the NF-κB:ER protein interaction [Stein and Yang, 1995]. It was also shown that internal deletion of the hinge region D did not impair the ability of ER to inhibit IL-6 promoter activation; however, it did diminish the ability of ER to bind p65 and p50 in vitro. Together with the results of Galien and Garcia [1997] and Liu et al. [2005], these data suggest that domains in the ERα responsible for binding to NF-κB may not be sufficient to inhibit the transcriptional activity. Our work further defines these interactions by demonstrating that binding of the ER with p50 requires a large region encompassing C–F, whereas the interaction of the ER with p65 can be conferred by a smaller region consisting of only EF. Importantly, despite the ability of the ER to bind p50 and p65 and inhibit NF-κB-responsive transcriptional activity and protein expression, we were unable to detect the ER in the NF-κB:κB-RE complex by supershift analysis. This could be due to alternate mechanisms of regulation such as that proposed by Ghisletti et al. [2005] who demonstrated that E2 inhibits NF-κB through prevention of p65 nuclear translocation. This may also explain why, despite the ability of the ER to bind to both p65 and p50, p65:p50 complexes appeared to be more sensitive to ERα overexpression than p50 homodimeric complexes. Moreover, the interaction between the ER and NF-κB response elements likely requires a larger complex involving co-regulators such as CBP [Nettles et al., 2008]. Thus, squelching of a limiting transcription factor or saturation of the target may explain why we did not always detect a dose–response to ERα expression.

In contrast to the mechanism of ER inhibition of NF-κB, the NF-κB proteins appear to directly interact with the ER:ERE complex. In the case of p50, a stable interaction occurs rendering it possible to supershift the ER:ERE complex with the p50 antibody. The interaction with p65 may be less stable such that it is not possible to supershift with anti-p65, however, the presence of p65 within this complex is highly probable since the p65 antibody strongly reduced the levels of the basal complex. Reduction of the protein:DNA complex may be due to destabilization as a result the antibody interaction with p65.

ER/NF-κB interactions have also been implicated in the progression of breast cancer. The progression of mammary epithelial cell line from ER+ non-malignant to ER− malignancy correlates with constitutive activation of NF-κB and IκB [Nakshatri et al., 1997]. Moreover, we have demonstrated that withdrawal of E2 from either MCF-7 cells in vitro or from MCF-7 tumors in ovariectomized nude mice results in activation of p50:p50 DNA binding [Pratt et al., 2003]. Thus, if ER represses both constitutive and inducible NF-κB activity, overexpression of NF-κB inducible genes in ER− cells may contribute to malignancy and resistance. ER+ breast cancer cells may lose their requirement for E2, possibly due, in part, to a corresponding increase in NF-κB activity, which provides growth and survival signals to the cells.

It is notable, however, that the introduction of the ER into LCC1 cells had relatively mild effect on NF-κB p50 homodimer DNA binding and on the expression of target genes such as p105 and

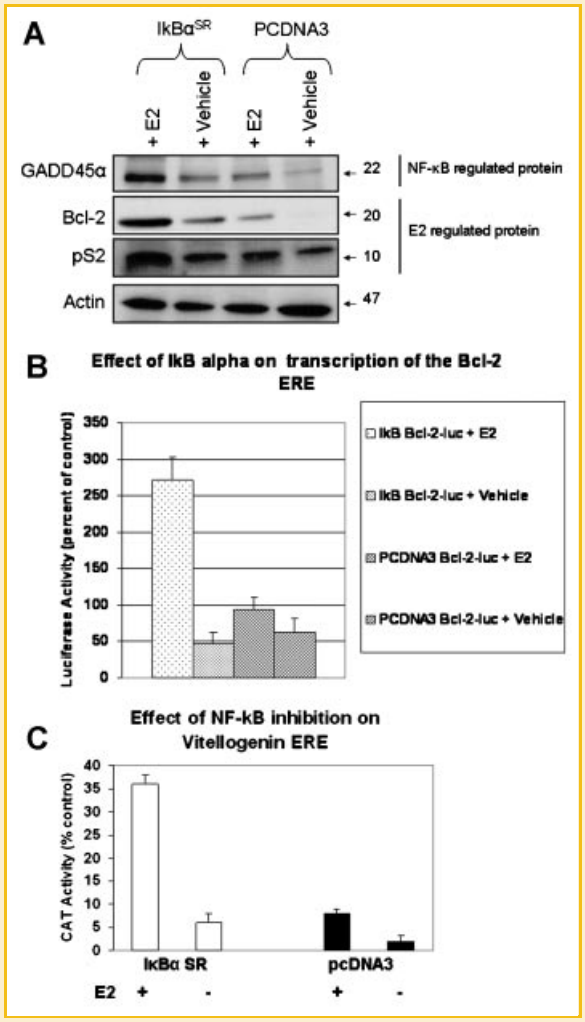

Fig. 7. Inhibition of NF-κB increases the expression of E2-regulated proteins. A: MCF-7 cells that were stably expressing a mutant IκBα super repressor ($IκBα^{SR}$) were maintained in phenol-red-free media for at least 4 days and treated with E2 (10^{-9} M) or vehicle for 4 days. Stable MCF-7 clones transfected with empty pcDNA3 vector were used as a control. Fifteen micrograms of whole-cell protein extract was subjected to immunoblot analysis of E2-regulated proteins. The expression of GADD45α was used to verify inhibition of NF-κB. Actin was used as an internal loading control. Molecular masses in kDa are shown. B: MCF-7 cells stably expressing and $IκBα^{SR}$ or pcDNA3 were transiently transfected with an expression vector containing two copies of the Bcl-2 ERE driving luciferase expression (Bcl-2-luciferase) or empty vector (pGL3-basic). C: Cells were also transfected with the vitellogenin ERE–chloramphenicol acetyl transferase (CAT) reported gene or control vector (pcDNA3). Results were normalized to internal controls as described in Materials and Methods Section. All experiments were performed in triplicate.

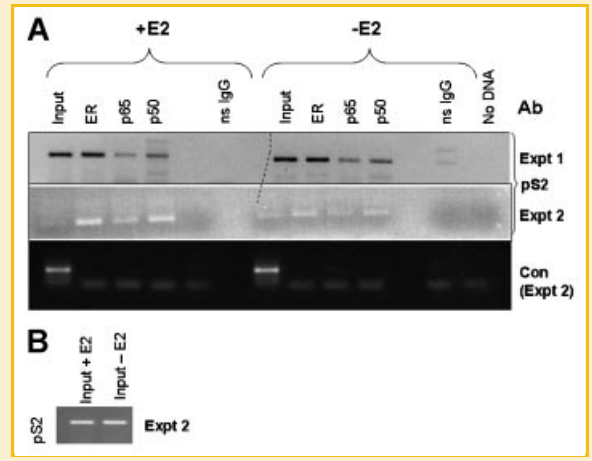

Fig. 8. ERα/NF-κB complex formation in vivo on the pS2 promoter. The status of the endogenous complex present on the estrogen responsive pS2 gene promoter region was determined using chromatin immunoprecipitation (ChIP). MCF-7 cells were grown in PRF media for 4 days and treated with either E2 (10^{-8} M) or vehicle for 45 min. Cells were processed as described in Materials and Methods Section, DNA was extracted and interactions with ERα, p50, and p65 were analyzed by PCR using a specific pair of primers spanning the ERE of the pS2 promoter. Two separate experiments are shown. A: Expt 1: 2 μl of ER, p50, p65, and normal IgG immunoprecipitation samples and input samples were used for PCR reactions. Expt 2: As in Expt 1 except 0.2 μl of input sample was used for PCR. Expt 2, bottom panel: Primers spanning a sequence within the estrogen receptor coding sequence were used as negative controls. Two microliters of all immunoprecipitation and input samples were used for PCR reactions. B: PCR of 2 μl of input was used from +estrogen (+E2) and −estrogen (−E2) samples from Expt 2.

ErbB2 than did the expression of the ER in ER− cells and yet it reduced proliferation in these cells. This may reflect several possible interactions involving the ER that predominate in LCC1 cells. Despite progression to hormone independence, LCC1 cells remain responsive to E2. Therefore, LCC1 cells may have adapted to use alternate mechanisms of regulation of at least some NF-κB target genes that would remain relatively unaffected by the introduction of the ER. This may be a necessary strategy by these cells given that they have fewer p65:p50 complexes than ER− cells. The proliferative signaling pathway used by LCC1 cells may alternate from NF-κB to ER relative to the presence of E2. In these cells, under these conditions, it is possible that overexpressed ER simply serves to squelch limiting transcription factors resulting in reduced proliferation.

Although loss of ER expression is sometimes observed in tamoxifen-resistant tumors, most tumors remain ER+ [Johnston et al., 1995]. Studies have also attempted to select for ER− sublines from ER+ T47D and MCF-7 breast cancer cells by growing them in the presence of tamoxifen [Graham et al., 1990; Montano and Katzenellenbogen, 1997], and long-term selection in the absence of estrogen [Katzenellenbogen et al., 1987; Murphy et al., 1990; Pink et al., 1996]. However, in the majority of cases, ER expression was maintained despite the conversion to a hormone-independent state [Fuqua, 2001]. In some instances, it is possible that hormone independence correlates not with loss of ER expression but with ER mutation. Given the possible ramifications on ERα conformation, interactions between the deleted form of the ER and NF-κB may be reduced. LCC1 cells are hormone independent yet continue to express the ERα. Sequencing analysis has shown, however, that they also express a 61 kDa variant of the ER corresponding to an exon 3 deletion as a result of alternate splicing (ERΔ3) [Han et al., 2004]. Exon 3 encodes the second zinc finger of the DNA-binding domain. Although the ERΔ3 protein is not able to bind EREs and does not activate transcription by directly binding DNA, it is capable of forming dimers with wild-type ER, which is also expressed in LCC1

cells. The fact that p50 was only able to interact with one form of the ER:ERE complex and not the other suggests that the ability of these proteins to bind one another is compromised and may help to explain the constitutive increase in NF-κB activity in these cells relative to parental MCF-7 cells.

Analysis of NF-κB-regulated genes involved in both proliferation and survival demonstrated clear inhibition in the ER− cells following expression of the ER both with and without E2.The one exception was cyclin D1. A canonical NF-κB-binding site is present within the cyclin D1 promoter [Karin et al., 2002] and cyclin D1 expression is elevated in 50% of breast cancers. This cyclin has been suggested to be an essential NF-κB target gene in normal mammary development and breast tumorigenesis [Joyce et al., 2001]. Importantly, the ERα also positively regulates cyclin D1 expression albeit from an atypical ERE [Altucci et al., 1996] so it is possible that while NF-κB-regulated expression of D1 is reduced, the ERα provides a positive transcriptional influence.

Both the ERα and NF-κB induce gene expression involved in growth and survival so the apparent incompatibility of these two signaling systems with respect to growth and survival in the ER− breast cancer cells is difficult to reconcile. Treatment of ER-transfected MDA-MB-231 cells with E2 does not induce c-myc and BRCA1/2 both known to be upregulated by E2 in ER+ cells [Lazennec and Katzenellenbogen, 1999] and E2 treatment of ER− cells ectopically expressing the ER results in growth inhibition and apoptosis [Jiang and Jordan, 1992; Lewis et al., 2005]. An important aspect of the present work is the demonstration that ER and NF-κB are mutually repressive and that in addition to p65, p50 interacts directly with the ERα in vitro and by co-IP from cells. We also show that both p65 and p50 associate with the ER:ERE complex and p65 and p50 are present on the promoter of estrogen-responsive pS2 promoter chromatin. This is contrary to the results of Nettles et al. [2008] who were unable to detect p65 on the pS2 promoter by ChIP. However, our results are in concert with the fact that p50 can be detected by supershift analysis on a canonical ERE.

Overall, our results suggest the following: first, the outcome of ERα expression in ER− cells may involve the inhibition of NF-κB-responsive genes. However, the ER does not confer proliferative signaling to these cells possibly due to the presence of high levels of nuclear NF-κB proteins in ER− cells which may prevent ectopically expressed ERα from activating at least a subset of E2-regulated genes. Moreover, E2 could alleviate NF-κB-mediated repression of apoptosis-inducing genes such as GADD45α as we observed here, the overall outcome being growth inhibition. Secondly, the acquisition of E2-independence in ER+ cells is associated with increased NF-κB and may be a consequence of ER mutation or alternative splicing. The induction of mainly p50:p50: Bcl-3 NF-κB activity [Pratt et al., 2003] could be a reflection of only partial signaling by this pathway in the presence of the ER, consequently allowing the cell to use either pathways depending on the presence of E2.

ACKNOWLEDGMENTS

This study was funded by grants from the Canadian Institutes for Health Research/Canadian Breast Cancer Research Alliance (CIHR) to M.A.C.P.

REFERENCES

Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, Weisz A. 1996. 17beta-estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. Oncogene 12:2315–2324.

Biswas DK, Dai S-C, Cruz A, Weiser B, Graner E, Pardee AB. 2001. The nuclear factor kappa B (NF-κB): A potential therapeutic target for estrogen receptor negative breast cancers. Proc Natl Acad Sci USA 98:10386–10391.

Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, Pardee AB, Inglehart JD. 2004. NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc Natl Acad Sci USA 101:10137–10142.

Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. 1993. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283–290.

Chen CL, Edelstein LC, Gelinas C. 2000. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol 20:2687–2695.

Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. 1997. Suppression of tumor-necrosis factor-induced cell death by inhibitor of apoptosis cIAP2 is under NF-κB control. Proc Natl Acad Sci USA 94:10057–10062.

Clarke R, Brunner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME, Dickson RB. 1989. Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both in vitro and in vivo. Proc Natl Acad Sci USA 10:3649–3653.

Cogswell PC, Scheinman RI, Baldwin AS, Jr. 1993. Promoter of the human NF-κB p50/p105 gene. Regulation by NF-κB subunits and by c-REL. J Immunol 150:2794–2804.

Eng FC, Barsalou A, Akutsu N, Mercier I, Zechel C, Mader S, White JH. 1998. Different classes of coactivators recognize distinct but overlapping binding sites on the estrogen receptor ligand binding domain. J Biol Chem 273: 28371–28377.

Evans MJ, Harris HA, Miller CP, Karathanasis SK, Adelman SJ. 2002. Estrogen receptors alpha and beta have similar activities in multiple endothelial cell pathways. Endocrinology 143:3785–3795.

Fuqua SA. 2001. The role of estrogen receptors in breast cancer metastasis. J Mammary Gland Biol Neoplasia 6:407–417.

Fuqua SA, Cui Y. 2004. Estrogen and progesterone receptor isoforms: Clinical significance in breast cancer. Br Cancer Res Treat 87 (Suppl 1): S3–S10.

Galien R, Garcia T. 1997. Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-κB site. Nucleic Acids Res 25:2424–2429.

Ghisletti S, Meda C, Maggi A, Vegeto E. 2005. 17beta-estradiol inhibits inflammatory gene expression by controlling NF-κB intracellular localization. Mol Cell Biol 25:2957–2968.

Gilmore TD. 1999. The Rel/NF-kappa B signal transduction pathway: Introduction. Oncogene 49:6842–6844.

Graham ML, Krett NL, Miller LA, Leslie KK, Gordon DF, Wood WM, Wei LL, Horwitz KB. 1990. T47DCO cells, genetically unstable and containing estrogen receptor mutations are a model for the progression of breast cancers to hormone resistance. Cancer Res 50:6208–6217.

Han F, Miksicek R, Clarke R, Conrad SE. 2004. Expression of an estrogen receptor variant lacking exon 3 in derivatives of MCF-7 cells with acquired estrogen independent or tamoxifen resistance. J Mol Endocrinol 32:935–945.

Higgins KA, Perez JR, Coleman TA, Dorshkind K, McComas WA, Sarmiento UM, Rosen CA, Narayanan R. 1993. Antisense inhibition of the p65 subunit of NF-κB blocks tumorigenicity and causes tumor regression. Proc Natl Acad Sci USA 90(21): 9901–9905.

Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. 1999. NF-κB function in growth control: Regulation of cyclin D1 expression and G0/G1-to-S phase transition. Mol Cell Biol 19:2690–2698.

Jeng MH, Jiang SY, Jordan VC. 1994. Paradoxical regulation of estrogen-dependent growth factor gene expression in estrogen receptor (ER)-negative human breast cancer cells stably expressing ER. Cancer Lett 82:123–128.

Jiang S-Y, Jordan VC. 1992. Growth regulation of estrogen receptor negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84:580–591.

Johnston SR, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M. 1995. Changes in estrogen receptor, progesterone receptor and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Res 55:3331–3338.

Jordan VC. 2004. Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell 3:207–213.

Joyce D, Albanese C, Steer J, Fu M, Bouzahzah B, Pestell RG. 2001. NF-kappaB and cell cycle regulation: The cyclin connection. Cytokine Growth Factor Rev 12:73–90.

Karin M. 1999. How NF-kappa B is activated: The role of the Ikappa B kinase (IKK) complex. Oncogene 49:6867–6874.

Karin M, Cao Y, Greten FR, Li ZW. 2002. NF-kappa B in cancer: From innocent bystander to major culprit. Nat Rev Cancer 2:301–310.

Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. 1987. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res 47:4355–4360.

Kitamura T, Sekimata M, Kikuchi S, Homma Y. 2005. Involvement of poly(ADP-ribose) polymerase I in ErbB2 expression in rheumatoid synovial cells. Am J Physiol (Cell Physiol) 289:C82–C88.

Klein-Hitpass L, Ryffel GU, Heitlinger E, Cato AC. 1988. A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucl Acids Res 16:647–663.

Lazennec G, Katzenellenbogen BS. 1999. Expression of human estrogen receptor using an efficient adenoviral gene delivery system is able to restore hormone-dependent features to estrogen receptor-negative breast carcinoma cells. Mol Cell Endocrinol 149:93–105.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. 2005. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759.

Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, DeLos Reyes A, Wing L, Jordan VC. 2003. Apoptotic action of 17-beta estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586–1597.

Liu H, Liu K, Bodenner DL. 2005. Estrogen receptor inhibits interleukin-6 gene expression by disruption of nuclear factor-κB transactivation. Cytokine 4:251–257.

Mader S, Chambon P, White JH. 1993. Defining a minimal estrogen receptor DNA binding domain. Nucleic Acid Res 21:1125–1132.

McGuire WL, Chamness GC, Fuqua SA. 1992. Abnormal estrogen receptor in clinical breast cancer. J Steroid Biochem Mol Biol 43:243–247.

McKay LI, Cidlowski JA. 1998. Cross-talk between nuclear factor-κB and the steroid hormone receptors: Mechanisms of mutual antagonism. Mol Endocrinol 12:45–56.

Montano MM, Katzenellenbogen BS. 1997. The quinone reductase gene: A unique estrogen receptor-regulated gene that is activated by antiestrogens. Proc Natl Acad Sci USA 94:2581–2586.

Murphy CS, Pink JJ, Jordan VC. 1990. Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. Cancer Res 50:7285–7292.

Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ, Sledge GW, Jr. 1997. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17:3629–3639.

Nettles KW, Gil G, Nowak J, Metivier R, Sharma VB, Greene GL. 2008. CBP is a dosage-dependent regulator of nuclear factor-κB suppression by the estrogen receptor. Mol Endocrinol 22:263–272.

Paya CV, Ten RM, Bessia C, Alcami J, Hay RT, Virelizier JL. 1992. NF-κB-dependent induction of the NF-κB p50 subunit gene promoter underlies its self-perpetuation of human immunodeficiency virus transcription in monocytic cells. Proc Natl Acad Sci USA 89:7826–7830.

Perillo B, Sasso A, Abbondanza C, Palumbo G. 2000. 17Beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. Mol Cell Biol 20:2890–2901.

Pink JJ, Bilimoria MM, Assikis J, Jordan VC. 1996. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer 74:1227–1236.

Pratt MAC, Bishop TE, White D, Yasvinski G, Menard M, Niu MY, Clarke R. 2003. Estrogen withdrawal-induced NF-κB activity and Bcl-3 expression in breast cancer cells: Roles in growth and hormone-independence. Mol Cell Biol 23:6887–6900.

Ray P, Ghosh SK, Zhang DH, Ray A. 1997. Repression of interleukin-6 gene expression by 17 beta-estradiol: Inhibition of the DNA binding activity of the transcription factors NF-IL6 and NF-κB by the estrogen receptor. FEBS Lett 409:79–85.

Sabbah M, Courilleau D, Mester J, Redeuilh G. 1999. Estrogen induction of the cyclin D1 promoter: Involvement of a cAMP response-like element. Proc Natl Acad Sci USA 96:11217–11222.

Stein B, Yang MX. 1995. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-κB and C/EBP beta. Mol Cell Biol 15:4971–4979.

Valentine JE, Kalkhoven E, White R, Hoare S, Parker MG. 2000. Mutations in the estrogen receptor ligand binding domain discriminate between hormone-dependent transactivation and transrepression. J Biol Chem 275:25322–25329.

Viatour P, Bentires-Alj M, Chariot A, Deregowski V, de Leval L, Merviall M-P, Bours V. 2003. NF-κB2/p100 induces Bcl-2 expression. Leukemia 17:1349–1356.

Wolf DM, Jordan VC. 1994. The estrogen receptor from a tamoxifen-stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Br Cancer Res Treat 1:129–138.

Zajchowski DA, Sager R, Webster L. 1993. Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res 53:5004–5011.

Zheng X, Zhang Y, Chen YQ, Castranova V, Shi X, Chen F. 2005. Inhibition of NF-κB stabilizes gadd45alpha mRNA. Biochem Biophys Res Comm 329:95–99.

Zhou Y, Yau C, Gray JW, Chew K, Dairkee SH, Moore DH, Eppenberger U, Eppenberger-Castori S, Benz CC. 2007. Enhanced NF-κB and AP-1 transcriptional activity associated with antiestrogen resistance breast cancer. BMC Cancer 7 59. 10.1186/1471-2407-7-59.