

Constitutive Activation of NF- κ B during Progression of Breast Cancer to Hormone-Independent Growth

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Breast cancers often progress from a hormone-dependent, nonmetastatic, antiestrogen-sensitive phenotype to a hormone-independent, antiestrogen- and chemotherapy-resistant phenotype with highly invasive and metastatic growth properties. This progression is usually accompanied by altered function of the estrogen receptor (ER) or outgrowth of ER-negative cancer cells. To understand the molecular mechanisms responsible for metastatic growth of ER-negative breast cancers, the activities of the transcription factor NF- κ B (which modulates the expression of genes involved in cell proliferation, differentiation, apoptosis, and metastasis) were compared in ER-positive (MCF-7 and T47-D) and ER-negative (MDA-MB-231 and MDA-MB-435) human breast cancer cell lines. NF- κ B, which is usually maintained in an inactive state by protein-protein interaction with inhibitor I κ Bs, was found to be constitutively active in ER-negative breast cancer cell lines. Constitutive DNA binding of NF- κ B was also observed with extracts from ER-negative, poorly differentiated primary breast tumors. Progression of the rat mammary carcinoma cell line RM22-F5 from an ER-positive, nonmalignant phenotype (E phenotype) to an ER-negative, malignant phenotype (F phenotype) was also accompanied by constitutive activation of NF- κ B. Analysis of individual subunits of NF- κ B revealed that all ER-negative cell lines, including RM22-F5 cells of F phenotype, contain a unique 37-kDa protein which is antigenically related to the RelA subunit. Cell-type-specific differences in I κ B α , - β , and - γ were also observed. In transient-transfection experiments, constitutive activity of an NF- κ B-dependent promoter was observed in MDA-MB-231 and RM22-F5 cells of F phenotype, and this activity was efficiently repressed by cotransfected ER. Since ER inhibits the constitutive as well as inducible activation function of NF- κ B in a dose-dependent manner, we propose that breast cancers that lack functional ER overexpress NF- κ B-regulated genes. Furthermore, since recent data indicate that NF- κ B protects cells from tumor necrosis factor alpha-, ionizing radiation-, and chemotherapeutic agent daunorubicin-mediated apoptosis, our results provide an explanation for chemotherapeutic resistance in ER-negative breast cancers.

Numerous experimental and clinical studies have established that estrogen plays a major role in the initiation and progression of breast cancers (37, 44, 49). Estrogen receptor (ER), which mediates the action of estrogen, is expressed in ~70% of breast cancers and is required for estrogen-dependent growth of breast cancers. As the cancer progresses, tumor cells acquire growth autonomy, no longer require estrogen, and become resistant to antiestrogens such as tamoxifen. Tumor heterogeneity and clonal selection of ER-negative cells, downregulation of ER expression, mutations of ER, altered regulation of ER-responsive genes, and generation of ER variants that function as dominant positive and estrogen-independent transcription factor are believed to be responsible for antiestrogen-resistant growth of breast cancers (9, 10, 28, 37, 58). These antiestrogen-resistant tumors are generally invasive and metastatic and respond poorly to chemotherapy and radiation treatment (10, 26).

Invasion and metastasis of cancer cells is a complex multi-step process that involves cell adhesion, proteolytic enzyme degradation of the extracellular matrix, and motility factors that influence cell migration (21, 32). Genes involved in this process are members of matrix metalloproteinase (MMP) fam-

ily, MMP activators such as urokinase plasminogen activator (uPA), MMP inhibitors such as tissue inhibitors of metalloproteinases, cell adhesion molecules such as integrin $\alpha_v\beta_3$, and angiogenic factors (7, 8, 21). Increased activity of MMPs has been observed during progression of breast cancer cell lines to an antiestrogen-resistant phenotype in vitro (16). Increased MMP2 activation has also been observed in established ER-negative breast cancer cell lines (3). However, the extent to which the loss of ER activity contributes to increased MMP activity is not well understood. One possibility is that ER has a negative influence on activities of transcription factors that upregulate the expression of MMPs and MMP activators such as uPA. AP-1, Ets, and NF- κ B are the three known transcription factors that are involved in expression of MMPs and uPA. For example, while Ets and AP-1 regulate the expression of interstitial collagenase and stromelysin I, NF- κ B and AP-1 regulate the expression of MMP9 and uPA (1, 20, 22, 31, 50, 65). Although two reports have suggested that the loss of ER activity is associated with increased AP-1 activity, the significance of this finding remains controversial, as two other reports have indicated that ER increases AP-1-dependent promoter activity (2, 16, 19, 61).

We focused our attention on NF- κ B activity for four reasons. First, NF- κ B regulates the expression of not only MMP9 and uPA but also integrin α_v , which potentiates metastasis by localizing MMP2 to the invasive surface of the cancer cells (8, 52). Second, vimentin, whose expression generally correlates with increased basement invasiveness and absence of ER in

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breast cancers, is regulated by NF- κ B (58, 4). Third, NF- κ B can indirectly increase the expression of AP-1-regulated genes by physically associating with transcription factor AP-1 (55). Fourth, NF- κ B is required for prevention of cell death induced by tumor necrosis factor alpha (TNF- α), ionizing radiation, or daunorubicin (a cancer chemotherapeutic compound), all of which are not effective in killing ER-negative breast cancer cells (6, 33, 62, 64, 66).

NF- κ B is a heterodimeric transcription factor which binds to decameric sequences present in the promoter regions of both cellular and viral genes (4, 5, 53, 63). The NF- κ B subunits, p50 (NF- κ B1, derived from proteolytic processing of its precursor p105) and RelA (p65), belong to the Rel family of proteins, which also includes p52 (NF- κ B2 and its precursor p100), RelB, the *v-rel* oncogene and *c-rel* proto-oncogene products, and the drosophila morphogen *dorsal*-encoded protein (5). The dimers containing c-Rel, RelA, and RelB can potently transactivate NF- κ B-dependent promoters, whereas homodimers of p50 and p52 subunit by themselves cannot, because of the absence of activation domains. In the majority of cells, NF- κ B exists in an inactive form in the cytoplasm bound to the inhibitory proteins referred to as I κ B. Several I κ Bs, including I κ B α (MAD3), I κ B β , p105/I κ B γ , p100/I κ B δ , I κ B ϵ , cactus, and relish, have been identified (5). Treatment of cells with various inducers result in degradation of I κ B proteins, thus releasing the bound NF- κ B, which translocates to the nucleus and up-regulates gene expression.

In this study, we have compared the constitutive and inducible DNA binding and transactivation properties of NF- κ B with hormone-dependent growth, ER status, and invasive and metastatic growth properties of breast cancer cell lines. A direct positive correlation of NF- κ B DNA binding and transactivation properties of NF- κ B to the acquisition of hormone-independent growth properties of a breast cancer cell line is presented. Although ligand-activated ER does not directly regulate the expression of any NF- κ B and I κ B proteins, the absence of ER appears to correlate with the presence of a novel protein of 37 kDa which is antigenically related to RelA. Furthermore, we show that ER can inhibit the constitutive as well as inducible activation function of NF- κ B in breast cancer cells. These findings along with recent reports of the role of NF- κ B in prevention of apoptosis provide insights into the mechanism of invasive and metastatic growth of hormone-independent breast cancers.

MATERIALS AND METHODS

Cell culture. MCF-7 and MDA-MB-231 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). T47-D was grown in RPMI 1640 supplemented with 10% FCS. MDA-MB-435 cells were grown in MEM containing 10% FCS, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acids, and vitamins. RM22-F5 cells were grown in LHC8 medium to obtain cells of the E phenotype (RM22-E) and in RPMI 1640 medium to obtain cells of the F phenotype (RM22-F) (39). All cell lines except RM22-F5 were obtained from the American Type Culture Collection. The RM22-F5 cell line was a generous gift from Antonino Passaniti (National Institute on Aging, Bethesda, Md.).

Plasmid constructs. Plasmid HEGO, encoding ER, and the expression vector pSG5, which was used to derive HEGO, were generous gifts from P. Chambon (IGBMC, Strasbourg, France) and were described previously (60). The NF- κ B/chloramphenicol acetyltransferase (CAT) reporter gene was a generous gift of E. O'Neill (Merck Research Laboratories, Rahway, N.J.) and contains four copies of NF- κ B binding site 5' GGGACTTTCC 3' and two copies of mutated NF- κ B site 5' GGGACTTTCT 3' upstream of the gamma fibrinogen basal promoter. NF- κ Bmut/CAT contains the sequence 5' ACTAACCTCA 3' instead of the NF- κ B binding site. Plasmid pcDNA3CAT was purchased from Invitrogen Corporation (San Diego, Calif.). I κ B ϵ cDNA was cloned from the MCF-7 cell line by reverse transcription-PCR and inserted into the *Eco*RI site of the pcDNA3 expression vector (Invitrogen). I κ B β cDNA was a generous gift from S. Ghosh (Yale University, New Haven, Conn.), and the cDNA was cloned into the *Not*I site of pcDNA3.

DNA transfections and CAT assays. Cells were split the day prior to transfection in culture medium and refed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS just before transfection. All transfections except ER cotransfection in the RM22-F cell line were performed by the calcium phosphate method. In addition to 5 μ g of CAT reporter genes, 2 μ g of β -galactosidase expression vector pCH110 (Pharmacia) was included in transfections to measure the transfection efficiency. The day after transfection, cells were refed with medium, and TNF- α (10 ng/ml) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 125 nM) was added. Alternatively, in some experiments, cells were maintained in DMEM throughout the transfection. Cells were harvested 36 h after transfection, and β -galactosidase activity was measured as described previously (40). CAT activity in equal numbers of β -galactosidase units from different cell lines was measured by using [¹⁴C]chloramphenicol and acetyl coenzyme A. Acetylated chloramphenicol was separated from nonacetylated chloramphenicol by thin-layer chromatography and quantitated by liquid scintillation counting. The results presented in graphs correspond to the averages of several independent transfection experiments. Neither CAT activity nor transfection efficiency was significantly affected by medium supplements (data not shown). Note that incubation of cells with either TNF- α or TPA, estrogen, and tamoxifen did not change the β -galactosidase activity in a given cell line (data not shown).

Western blotting and immunoprecipitation. Antibodies against NF- κ B and I κ B proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.) and Upstate Biotechnology (Lake Placid, N.Y.). ER antibody was a generous gift of P. Chambon. Cell extracts were prepared in radioimmunoprecipitation assay buffer (23) or in whole-cell extraction buffer (for tumor samples; see below) and subjected to Western blotting and immunoprecipitation as recommended by the antibody manufacturers. In immunoprecipitation experiments, lysate from cells that had been incubated with Trans ³⁵S-label (ICN) for 4 h was used in a standard assay (23).

EMSA. Whole-cell extracts from cell lines and tumors for electrophoretic mobility shift assays (EMSAs) were prepared as described by Schulze-Osthoff et al. (51), and 6 μ g of extract was subjected to EMSA in a reaction buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 0.5 mM dithiothreitol, 2 μ g of poly(dI-dC) · poly(dI-dC), 100 mM KCl, and 10% glycerol as described previously (51). Oligonucleotide probes containing consensus sequences for NF- κ B, AP-1, SP1, and CTF/NF-1 binding sites were purchased from Promega Corporation (Madison, Wis.).

RESULTS

Constitutive DNA binding activity of NF- κ B in hormone-independent breast cancer cell lines. Breast cancer cell lines were examined for NF- κ B DNA binding activity by EMSA. Cell lines for this study were chosen in such a way that the results could be correlated with ER status and hormone requirement for growth. Human breast cancer cell lines studied were MCF-7 and T47-D (both ER positive and hormone dependent) and MDA-MB-231, MDA-MB-435, and MDA-MB-468 (all ER negative and hormone independent [54]). In addition, NF- κ B DNA binding activity in a well-differentiated hormone-dependent, ER-positive rat mammary adenocarcinoma cell line, RM22-F5, which can be converted from hormone-dependent, epithelial cell-like phenotype (E phenotype) to hormone-independent, fibroblast-like, spindle carcinoma phenotype (F phenotype) by altering growth conditions was also examined to determine whether progression of breast cancer from hormone-dependent phenotype to hormone-independent phenotype is accompanied by changes in NF- κ B DNA binding activity (39).

NF- κ B DNA binding activity obtained with whole-cell extracts from breast cancer cell lines is shown in Fig. 1A. ER levels in these extracts are shown in Fig. 1B. Among human breast cancer cell lines examined, MDA-MB-231 contained the highest level of NF- κ B DNA binding activity, followed by MDA-MB-468, MDA-MB-435, T47-D, and MCF-7 (compare lanes 1 to 5). A twofold increase in NF- κ B DNA binding activity was observed when RM22-F5 was converted from ER-positive (RM22-E) phenotype to ER-negative (RM22-F) phenotype (lanes 6 and 7). Similar patterns of NF- κ B DNA binding activity were observed in all cell lines except MDA-MB-435 when nuclear extract instead of whole-cell extract was used in EMSAs (data not shown). The reason for this discrepancy in MDA-MB-435 is not known. It could be due either to technical

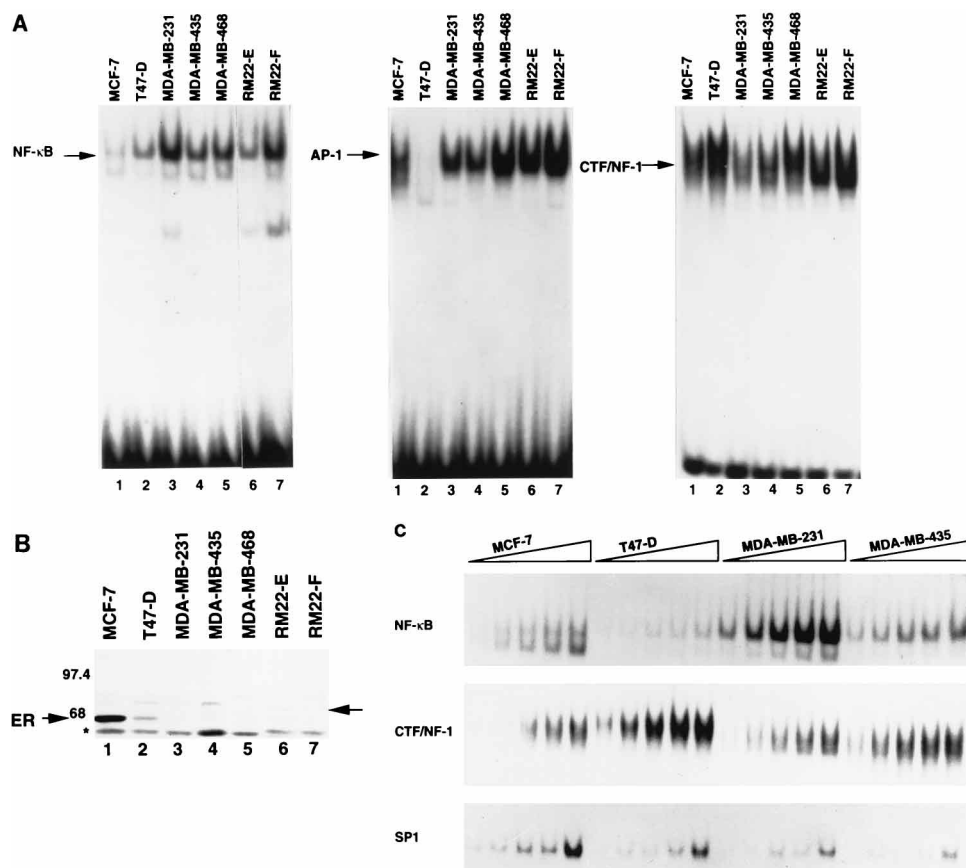


FIG. 1. ER-positive and ER-negative breast cancer cells contain different levels of NF- κ B DNA binding activity. (A) DNA binding activities of various transcription factors in established breast cancer cell lines. Whole-cell extracts (6 μ g) from ER-positive (lanes 1, 2, and 6) and ER-negative (lanes 3 to 5 and 7) cell lines were incubated with double-stranded oligonucleotides containing either an NF- κ B binding site (left panel), AP-1 binding site (middle panel), or CTF/NF-1 binding site (right panel) and subjected to EMSA. Specific DNA-protein complexes are indicated by arrows. (B) Analysis of ER in various breast cancer cell lines. Whole-cell extracts (100 μ g) from the indicated cell lines were analyzed for ER by Western blotting. ER is indicated by arrows. A nonspecific interaction of antibody is indicated by an asterisk. Sizes are indicated in kilodaltons. (C) Comparative analysis of NF- κ B, CTF/NF-1, and SP1 in various breast cancer cell lines. NF- κ B, CTF/NF-1, and SP1 probes were incubated with increasing quantities of whole-cell extracts (1.5, 3.0, 4.5, 6.0, and 7.5 μ g) from the indicated cell lines and subjected to EMSA. Only DNA-protein complexes are shown.

difficulties in retaining intact nuclei during extraction procedure or to the fact that DNA binding-competent NF- κ B is localized in the cytoplasm of these cells. In immunofluorescence studies, 15 to 20% of MDA-MB-231, MDA-MB-435, and RM22-F cells displayed nuclear staining of the RelA subunit of NF- κ B (data not shown).

In agreement with previously published reports of an increase in transcription factor AP-1 DNA binding activity during progression of MCF-7 to antiestrogen-resistant phenotype (2, 16), ER-negative cell lines, in general, contained elevated levels of AP-1 (Fig. 1A, AP-1; compare lanes 1 and 2 with lanes 3 to 5). For unknown reasons, T47-D appears to lack transcription factor AP-1 (AP-1, lane 2). In contrast to AP-1 and NF- κ B, there was no correlation between transcription factor CTF/NF-1 DNA binding and ER status in breast cancer cell lines. In fact, T47-D appears to contain more CTF/NF-1 than MDA-MB-231 and MDA-MB-435 (Fig. 1A, CTF/NF-1; compare lanes 1 to 4). These results suggest that the increased DNA binding activity of NF- κ B and AP-1 in ER-negative cell lines is not due to a global increase in transcription factors as a consequence of lack of ER expression. The NF- κ B-DNA complex in ER-negative cell lines is comprised of the RelA subunit of NF- κ B, as RelA antibody but not c-Rel antibody supershifted this complex (data not shown).

To further demonstrate that ER-negative breast cancer cell lines contain more constitutive NF- κ B DNA binding activity than ER-positive cell lines, titration experiments using CTF/NF-1 and SP1 as internal controls were performed. Radiolabeled NF- κ B, CTF/NF-1, and SP1 probes were incubated with increasing amount of whole-cell extracts from MCF-7, T47-D, MDA-MB-231, and MDA-MB-435 cell lines and subjected to EMSA. As in Fig. 1A, a higher level of NF- κ B DNA binding activity was observed in ER-negative cell lines compared to ER-positive cell lines (Fig. 1C). For example, when SP1 DNA binding activity is considered equal in all cell lines, the highest level of NF- κ B is in MDA-MB-231, followed by MDA-MB-435, T47-D, and MCF-7. Similarly, if CTF/NF-1 DNA binding activity is considered equal in all cell lines, NF- κ B DNA binding activity is highest in MDA-MB-231, followed by MDA-MB-435, MCF-7, and T47-D.

In view of the inverse correlation between ER level and NF- κ B DNA binding activity in established cell lines, whole-cell extracts prepared from primary breast tumors were also examined for NF- κ B DNA binding activity and for the presence of ER. Extracts from three of six tumors displayed NF- κ B DNA binding activity (Fig. 2A; compare lanes 2 to 7). Note that ER is present in tumors that lack NF- κ B DNA binding activity (Fig. 2B). These results indicate that constitutive

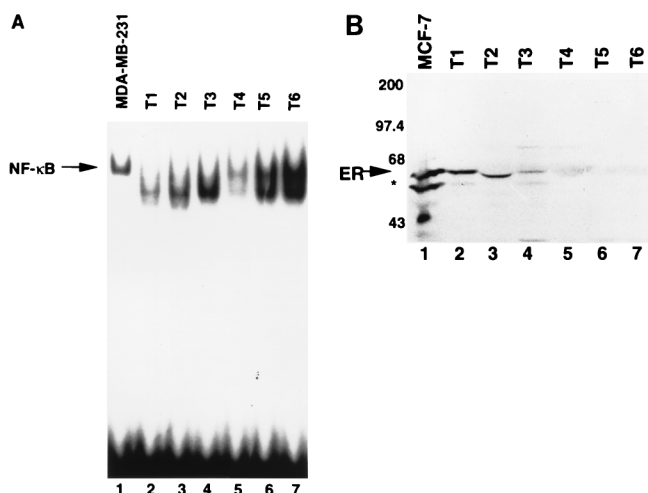


FIG. 2. NF- κ B DNA binding activity in primary breast cancers. (A) Primary breast cancers display constitutive NF- κ B DNA binding activity. Lysates from tumors (6 μ g) were subjected to EMSA. (B) Tumors with constitutive NF- κ B DNA binding activity lack ER. Lysates used in EMSA (100 μ g) were analyzed for ER level by Western blotting. The asterisk is as in Fig. 1. Sizes are indicated in kilodaltons.

NF- κ B DNA binding activity is not restricted to cultured cell lines but is found in primary breast tumors as well and therefore might have some significance with respect to disease progression.

Differential expression of RelA in ER-positive and ER-negative breast cancer cell lines. The elevated NF- κ B DNA binding in ER-negative cell lines could be due to either increased expression of NF- κ B subunits or reduced expression of I κ Bs in these cells. As a first step in distinguishing between these two possibilities, NF- κ B binding activity in detergent-treated cytoplasmic extracts was analyzed. DNA binding activity was highest in the cytoplasmic extracts of MDA-MB-435, followed by MCF-7, MDA-MB-468, MDA-MB-231, and T47-D (data not shown). Since the amount of NF- κ B retained in the cytoplasm did not correlate with the ER level, the possibility of differential expression of individual subunits of NF- κ B and I κ Bs was examined by Western blotting. In addition, as there was an inverse correlation between NF- κ B DNA binding activity and ER level, a possible effect of estradiol on the level of I κ Bs and NF- κ B subunits was also examined in parallel.

All of the human breast cancer cell lines examined contained similar levels of the RelA subunit with the expected molecular mass of 65 kDa, and estradiol had no effect on the RelA protein level (Fig. 3A, top panel). However, RelA-specific antibody (raised against amino acids 3 to 19) strongly reacted with an additional protein of 37 kDa [referred as p37(RelA) hereafter] in MDA-MB-231 and MDA-MB-435 (Fig. 3A, top panel). MDA-MB-231 contains more p37(RelA) than MDA-MB-435 (compare lanes 5 to 8). An antibody against amino acids 276 to 502 of RelA (Upstate Biotechnology) also reacted with this 37-kDa protein in the MDA-MB-231 cell line (data not shown). There was no significant difference in levels of p50 among various cell lines, although processing of p105 to p50 and p100 to p52 appears to be more efficient in ER-positive cell lines (Fig. 3A, middle and bottom panels). Also note that p105 runs as a doublet in MDA-MB-231 and MDA-MB-435. Like RelA protein, estradiol had no effect on p50 and p52. In agreement with the results of Dejardin et al. (11), the overall level of p105 appears to be higher in ER-negative cell lines (Fig. 3A, middle panel).

A remarkable cell-type-specific variation in I κ B protein level was observed (Fig. 3B). For example, expression of I κ B α was low in MDA-MB-435 (top panel, lanes 7 and 8). In contrast, expression of I κ B β was low in MDA-MB-231 (middle panel, lanes 5 and 6). The difference in I κ B β level among untreated and estradiol-treated MCF-7 cells is not reproducible (middle panel, lanes 1 and 2). I κ B γ antibody reacted with a 105-kDa protein in MDA-MB-435 and a 70-kDa protein in MCF-7, T47-D, and MDA-MB-231 (lower panel). Interestingly, the same antibody also recognized two proteins of \sim 40 kDa [referred to as p40(I κ B γ) hereafter] in MCF-7 and T47-D (bottom panel, lanes 1 to 4). A reduced level of a slowly migrating form of p40(I κ B γ) was detected in MDA-MB-231 but not in MDA-MB-435 (bottom panel, lanes 5 to 8).

Recent clinical studies have indicated that ER-negative breast cancers originate either from an ER-positive tumor or directly from ER-negative mammary luminal epithelial cells (28, 41). As there are no genetic markers that can distinguish these two types of ER-negative breast cancers, it is extremely difficult to determine which of the specific differences in gene expression pattern among ER-positive and ER-negative breast cancer cell lines reflect differences in cell genotype rather than change associated with malignancy. RM22-E and RM22-F cells are better suited for distinguishing between these two possibilities, as any differences in NF- κ B and I κ B proteins between these cell types should reflect genetic changes associated with progression to ER-negative phenotype. Unlike for human breast cancer cell lines, there was no difference in the levels of p105, I κ B α , and I κ B β between RM22-E and RM22-F (Fig. 3C, lanes 1, 2, and 5 to 8). Interestingly, however, RM22-F but not RM22-E contained p37(RelA) protein (Fig. 3C, lanes 3 and 4). Neither RM22-E nor RM22-F contained I κ B γ and p52, and the two cell types contained equal amounts of p100 (data not shown). Taken together, the results indicate that the expression of p37(RelA) is common to all three ER-negative breast cancer cell lines examined and that its expression alone correlates with constitutive DNA binding of NF- κ B.

To further explore the relationship between p37(RelA) and RelA protein, immunoprecipitation using RelA antibody was performed. Immunoprecipitations were performed in radioimmunoprecipitation assay buffer (see Materials and Methods) so as to minimize nonspecific antigen-antibody interactions. p37(RelA) was efficiently immunoprecipitated from the lysates of MDA-MB-231 and RM22-F cells, and the ratios between RelA and p37(RelA) in these cells in both assays (Western blotting and immunoprecipitation) were almost identical (Fig. 3D; compare lanes 2, 4, 6, and 8). Note that a 43-kDa protein in MCF-7 which cross-reacted with the RelA antibody in Western blotting was not immunoprecipitated. Unexpectedly, p40(I κ B γ), which was recognized by I κ B γ antibody in Western blotting and appears to be fairly abundant in MCF-7 and T47-D, could not be immunoprecipitated (data not shown). Whether p40(I κ B γ) is a member of I κ B family or an unrelated protein carrying an epitope recognized by the antibody remains to be investigated. Note that the I κ B γ antibody used in our study has been shown to recognize a 37-kDa protein in lymphoid cell lines (27).

Constitutive activities of NF- κ B-dependent promoters in ER-negative breast cancer cell lines. A recent study indicated that the activation of NF- κ B-dependent genes by certain extracellular signals requires not only nuclear translocation of NF- κ B but also a tyrosine kinase-dependent transactivation process, and an increased DNA binding need not necessarily correlate with increased expression of NF- κ B-dependent promoters (68). This finding prompted us to investigate whether persistent DNA binding activity of NF- κ B in ER-negative cell

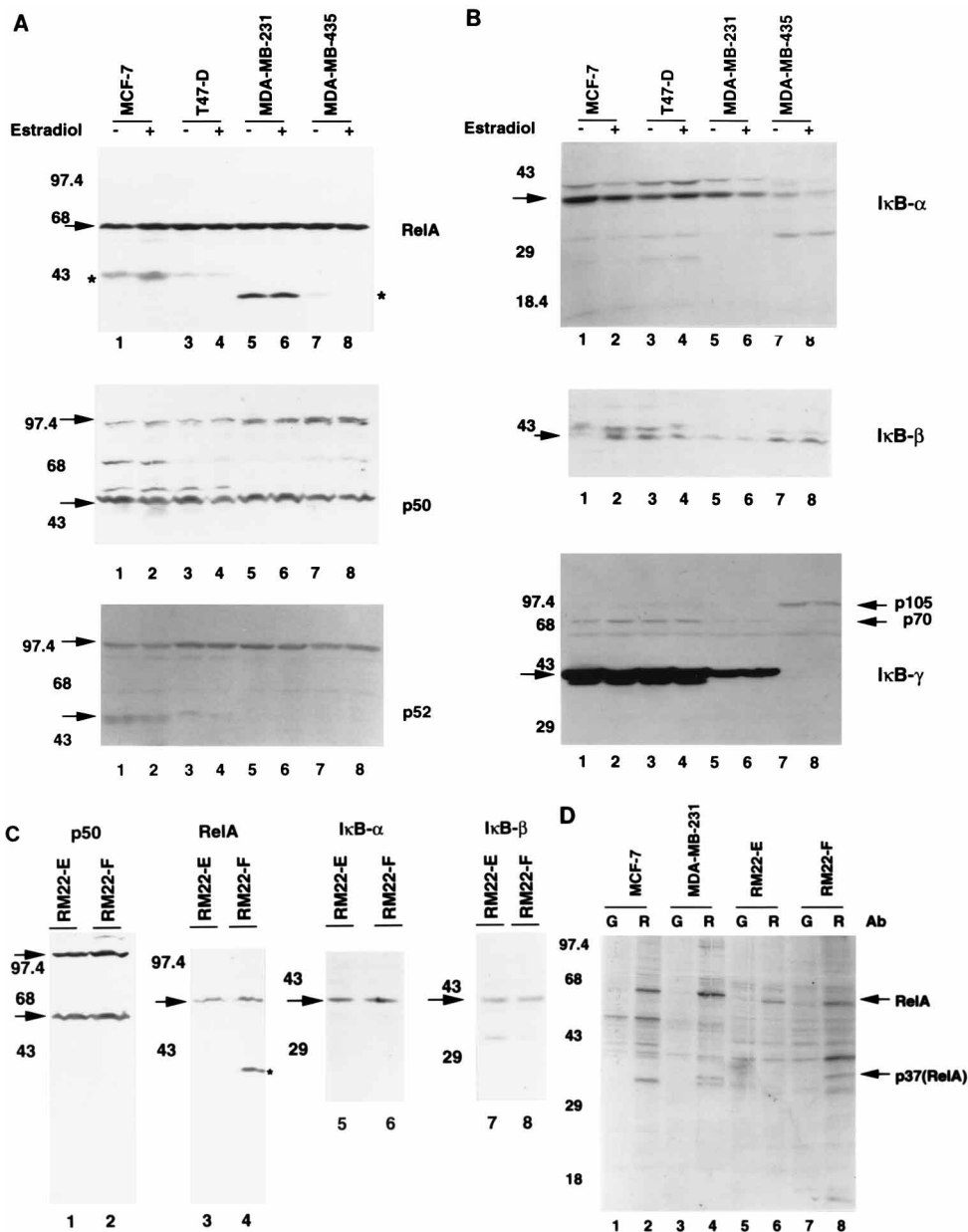
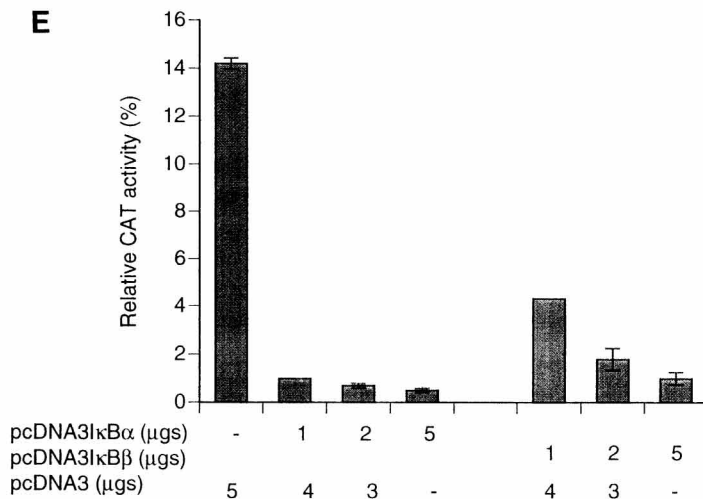
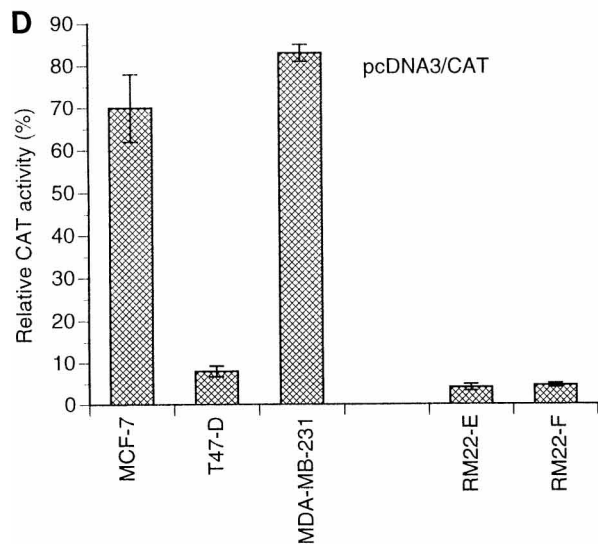
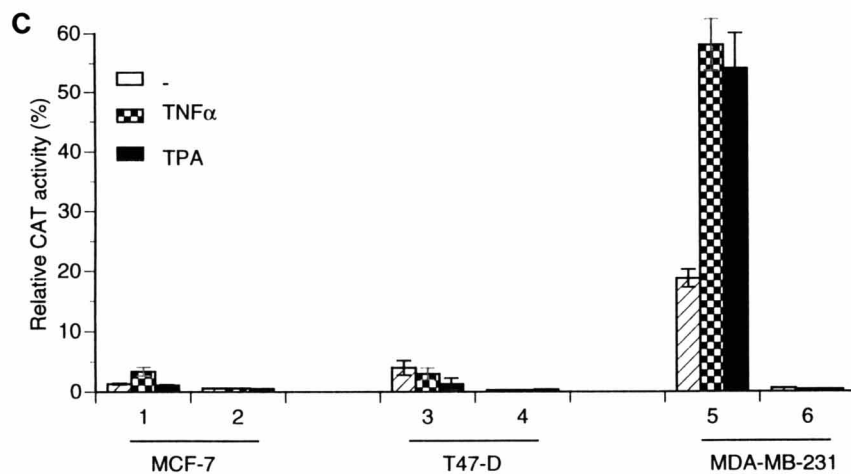
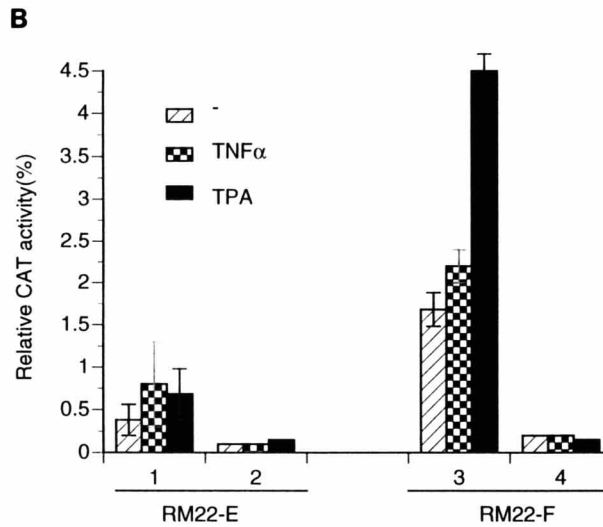
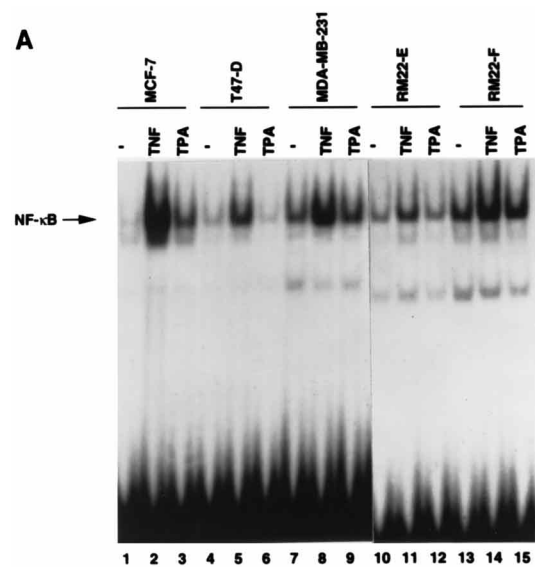


FIG. 3. Differential expression of NF- κ B and I κ B family proteins in ER-positive and ER-negative cells. (A) p37(RelA) is expressed in ER-negative cell lines. Whole-cell extracts (100 μ g) from the indicated cell lines with (+) or without (-) 17 β -estradiol treatment (10^{-8} M for 24 h) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using antibodies against RelA (top panel), p50 (middle panel), and p52 (bottom panel). RelA protein is indicated by an arrow, and additional cell-type-specific cross-reacting proteins are indicated by asterisks. p50, p52, and their precursors (p105 and p100, respectively) are indicated by arrows. Note that p37(RelA) (indicated by an asterisk on the right) is present only in MDA-MB-231 and MDA-MB-435 cell extracts. Positions of molecular weight standards are in kilodaltons shown on the left. (B) I κ B expression in breast cancer cell lines. Whole-cell extracts from the indicated cell lines were subjected to Western blotting using antibodies against I κ B α , I κ B β , and I κ B γ . I κ B α , I κ B β , wild-type I κ B γ , and p105, which shares its C terminus with I κ B γ , are indicated by arrows. In ER-positive cells, I κ B β and p40(I κ B γ) appear as a doublet. Note the low abundances of I κ B α and I κ B β in MDA-MB-435 and MDA-MB-231, respectively. The difference in I κ B β level in untreated and estradiol-treated MCF-7 is not reproducible. (C) p37(RelA) is made only after progression of RM22-F5 cells from ER-positive phenotype (RM22-E) to ER-negative phenotype (RM22-F). Whole-cell extracts from RM22-E and RM22-F cell lines were analyzed by Western blotting using the indicated antibodies. p37(RelA) protein in RM22-F is indicated by an asterisk. (D) p37(RelA) can be immunoprecipitated from MDA-MB-231 and RM22-F. Lysates from cells labeled with Trans 35 S-label for 4 h were immunoprecipitated with either glutathione S-transferase antibody (Ab) (lanes G) or RelA antibody (lanes R) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Wild-type RelA and p37(RelA) proteins are indicated.

lines is correlated with increased expression of NF- κ B-dependent promoters. Experiments were also performed with ER-positive cell lines where the NF- κ B DNA binding activity was elevated to a level similar to or higher than that of ER-negative cell lines. The rationale behind this experimental strategy is that if ER-positive cell lines differ from ER-negative cell lines

with respect to NF- κ B DNA binding activity but not in trans-activation potential, increasing the DNA binding activity of NF- κ B by treating cells with an agent such as TNF- α or the tumor promoter TPA should increase NF- κ B-dependent promoter activity in ER-positive cell lines. As shown in Fig. 4A, TNF- α -treated MCF-7 displayed more NF- κ B DNA binding



activity than untreated and TNF- α -treated MDA-MB-231. NF- κ B DNA binding activities in TPA-treated MCF-7, TNF- α -treated T47-D, untreated MDA-MB-231, TNF- α -treated RM22-E, and untreated RM22-F were almost identical (compare lanes 3, 5, 7, 11, and 13). Cells were treated with these reagents for 12 h, since at this time point, cells were apparently healthy and the reagents did not unduly influence the expression of an internal control plasmid used to monitor transfection efficiency of different cell lines. Also, at this time point, the kinetics of TNF- α -mediated NF- κ B binding and I κ B α degradation in MCF-7 and MDA-MB-231 were identical (data not shown). Since each of the cell lines used in this study requires a specific medium for optimal growth and medium supplements may inadvertently affect both transfection efficiency and activities of transfected promoters, all cells were maintained in DMEM supplemented with 10% FCS at the time of transfection. Cells were refed with either culture medium or DMEM-10% FCS 24 h after transfection with or without inducing agents. Neither transfection efficiency nor promoter activity was unduly influenced by cell line-specific medium supplements (data not shown). MDA-MB-435 was excluded from this analysis due to very poor transfection efficiency compared to other cell lines and difficulties in obtaining reproducible results.

Activity of a minimum promoter-CAT reporter (68-bp fibrinogen basal promoter encompassing the TATA box and an SP1 binding site) containing multiple NF- κ B binding sites (NF- κ B/CAT; see Materials and Methods) in RM22-F5 is shown in Fig. 4B. In RM22-E, the transfected promoter displayed ~2-fold-higher activity than a similar reporter containing mutated NF- κ B DNA binding sites (compare lanes 1 and 2). In contrast, 6- to 10-fold-higher activity of NF- κ B/CAT compared to NF- κ Bmut/CAT was observed in the RM22-F cell line (lanes 3 and 4). A modest increase in reporter activity was observed in both cell lines upon TNF- α treatment. In contrast, TPA increased the activity of the reporter mostly in the RM22-F cell line (Fig. 4B). Note that the activities of a similar reporter containing the cytomegalovirus immediate-early gene enhancer-promoter were almost the same in the two cell types (Fig. 4D).

Similar analysis in human breast cancer cell lines is shown in Fig. 4C. Among various cell lines, the activity of the NF- κ B/CAT reporter was lowest in MCF-7 (compare lanes 1 and 2). A twofold-higher activity was seen in T47-D compared to MCF-7, and this activity decreased upon TNF- α and TPA treatment (lanes 3 and 4). In comparison with MCF-7 and T47-D, the activity of NF- κ B/CAT was considerably higher in MDA-MB-231, and this activity was further enhanced upon TNF- α and TPA treatment (lanes 5 and 6). Note the striking correlation between constitutive NF- κ B DNA binding activity in various cell types to the promoter activity and the inverse correlation between ER level and NF- κ B/CAT activity. Also, as with the RM22-E cell line, the promoter was not propor-

tionally active in both MCF-7 and T47-D when NF- κ B DNA binding activity was elevated to the level in MDA-MB-231. For example, levels of NF- κ B DNA binding in TPA-treated MCF-7 and MDA-MB-231 cell lines are identical, yet TPA did not increase NF- κ B/CAT activity in MCF-7 to the level in MDA-MB-231 (Fig. 4C, lanes 1 and 5). In the TNF- α -treated MCF-7 cell line, although NF- κ B DNA binding activity is much higher than that in MDA-MB-231 (Fig. 4A), NF- κ B/CAT displayed very little activity compared to MDA-MB-231. The difference between MCF-7 and MDA-MB-231 is specific to the NF- κ B/CAT reporter, as the pcDNA3/CAT reporter was equally active in MCF-7 and MDA-MB-231 (Fig. 4D). These results suggest that ER-positive and ER-negative cell lines differ not only with respect to the level of constitutive NF- κ B DNA binding activity but also in the ability to support gene activation by NF- κ B.

To test whether the constitutive activity in the MDA-MB-231 cell line can be repressed by inhibiting NF- κ B DNA binding, the NF- κ B/CAT gene was cotransfected with an expression vector for either I κ B α or I κ B β . Both expression vectors efficiently repressed the NF- κ B/CAT reporter activity in the MDA-MB-231 cell line (Fig. 4E), suggesting that the activity of NF- κ B/CAT reporter is NF- κ B dependent.

ER represses the constitutive activities of NF- κ B-dependent promoters in MDA-MB-231 and RM22-F cell lines. The lack of NF- κ B/CAT reporter activity in ER-positive cell lines, even after induction of NF- κ B DNA binding activity by TPA and TNF- α treatment, suggests the following: (i) ER interferes with the transcriptional activation function of NF- κ B; and (ii) a posttranslational modification of NF- κ B is essential for optimal transcriptional activation by NF- κ B, and such modification takes place only in ER-negative cell lines. To address this issue, the MDA-MB-231 cell line was transfected with the NF- κ B/CAT reporter along with increasing concentrations of an ER expression vector. At low ER expression vector concentrations, efficient repression of NF- κ B/CAT activity was observed in the presence of estrogen (Fig. 5A). However, at higher ER expression vector concentrations, promoter activity decreased by 80% even without estrogen treatment. It is interesting that the antiestrogen tamoxifen did not block the repressor function of ER (Fig. 5A). In contrast to the NF- κ B reporter gene, pcDNA3/CAT was activated modestly by cotransfected ER (Fig. 5A). These results suggest that inhibition of NF- κ B promoter activity by ER is not due to an adverse effect of ER on cell growth. As with the MDA-MB-231 cell line, ER inhibited NF- κ B/CAT activity in the RM22-F cell line (Fig. 5B).

To evaluate whether unliganded ER can inhibit TNF- α - and TPA-mediated NF- κ B activation, MDA-MB-231 cells were cotransfected with ER and the NF- κ B/CAT reporter and treated with either TNF- α or TPA. Efficient repression of TNF- α - and TPA-mediated activation of the NF- κ B/CAT reporter was observed in ER-transfected cells (Fig. 5C). From these results, we

FIG. 4. Constitutive activities of NF- κ B-dependent promoters in ER-negative breast cancer cell lines. (A) Induction of NF- κ B DNA binding activity by TNF- α and TPA in ER-positive and ER-negative breast cancer cell lines. Cells were treated with either 10 ng of TNF- α per ml (lanes 2, 5, 8, 11, and 14) or 125 nM TPA (lanes 3, 6, 9, 12, and 15) for 12 h, and lysates were assayed for NF- κ B DNA binding. (B) Activities of NF- κ B-dependent promoters in RM22-E and RM22-F cell lines. Cells were transfected with 5 μ g of either NF- κ B/CAT (lanes 1 and 3) or NF- κ Bmut/CAT (lanes 2 and 4) reporter gene along with an internal control plasmid coding for β -galactosidase. After 24 h of transfection, cells were fed with either regular medium (-) or medium supplemented with TNF- α (10 ng/ml) or TPA (125 nM). CAT activity in equal numbers of β -galactosidase units (30 U) was measured as described in Materials and Methods. The values correspond to percent conversion of chloramphenicol to the acetylated form. (C) Activities of the NF- κ B/CAT reporter (lanes 1, 3, and 5) and NF- κ Bmut/CAT reporter (lanes 2, 4, and 6) in human breast cancer cell lines. Experiments were performed as for panel B. (D) Activities of a cytomegalovirus enhancer-promoter-containing CAT reporter (pcDNA3CAT) in various cell lines. Five micrograms of pcDNA3CAT was transfected along with a β -galactosidase vector as described above and assayed for CAT activity. The values represent percent conversion of chloramphenicol to the acetylated form. (E) I κ B α and I κ B β repress constitutive activity of the NF- κ B/CAT reporter in the MDA-MB-231 cell line. Cells were transfected with 5 μ g of NF- κ B/CAT reporter along with indicated amount of expression vector and 2 μ g of control plasmid coding for β -galactosidase. CAT activity was measured 48 h after transfection.

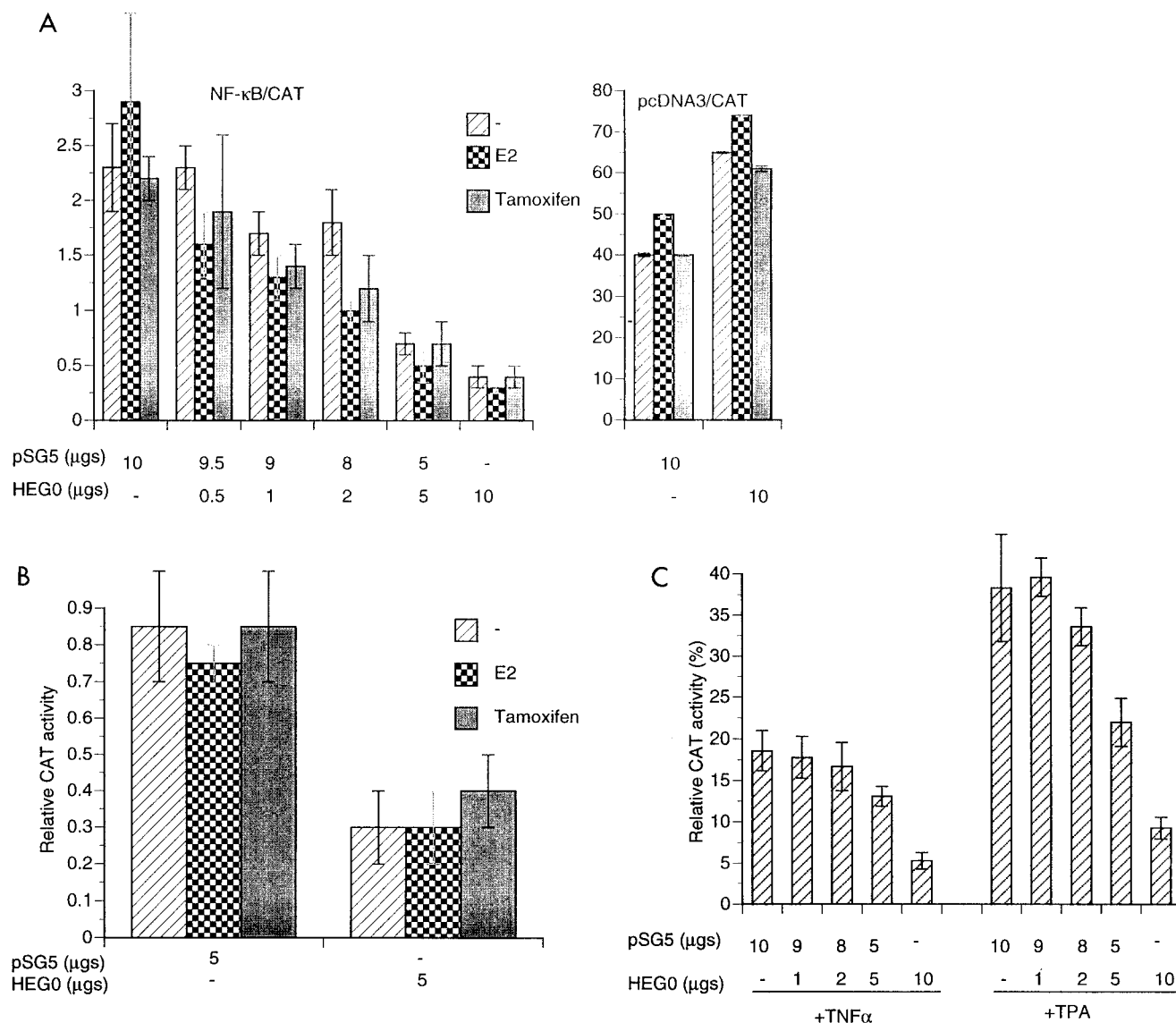


FIG. 5. Constitutive and inducible activations of the NF- κ B/CAT reporter are repressed by ER. (A) Effect of ER on the constitutive activity of NF- κ B/CAT in the MDA-MB-231 cell line. The MDA-MB-231 cell line was transfected with the indicated quantity of empty expression vector pSG5 and pSG5 containing coding sequences for ER (HEGO). After 24 h of transfection, cells were treated with either 10^{-8} M 17 β -estradiol (E2) or 10^{-6} M tamoxifen or left untreated (-). CAT assays were performed 36 h after transfection. The effect of cotransfected ER (10 μ g) on the activity of a cytomegalovirus enhancer-promoter-containing CAT reporter (pcDNA3/CAT) was also measured as a control. (B) Effect of ER on the constitutive activity of the NF- κ B/CAT gene in the RM22-F cell line. The RM22-F cell line was transfected with 1 μ g of NF- κ B/CAT reporter and 5 μ g of HEGO, using SuperFect transfection reagent (Qiagen, Chatsworth, Calif.). Cells were treated as for panel A, and CAT activity was measured 36 h after transfection. (C) Effect of ER on TNF- α - and TPA-inducible expression of the NF- κ B/CAT reporter gene. MDA-MB-231 cells were transfected with 5 μ g of NF- κ B/CAT and the indicated amount of ER expression vector. Cells were treated with TNF- α (10 ng/ μ l) or TPA (125 nM) 24 h after transfection. CAT activity was measured 36 h after transfection. The values represent percent conversion of chloramphenicol to the acetylated form.

suggest that ER plays a dominant role in limiting the transcription response through NF- κ B even when DNA binding of NF- κ B is activated by extracellular signals and that this function of ER may be responsible for the poor activation of the NF- κ B/CAT reporter upon treatment of the MCF-7 cell line with TNF- α and TPA (Fig. 4C).

DISCUSSION

In this report, we present evidence for constitutive activation of NF- κ B in ER-negative breast cancer cell lines and show that this activity is due in part to lack of functional ER. Moreover, constitutive DNA binding of NF- κ B was also observed in three

of three ER-negative breast cancer samples examined. Strikingly, all of these tumors are histologically poorly differentiated infiltrating ductal carcinomas with lymph node metastasis. Although further studies using additional samples are required, the present results are encouraging enough to suggest a link between constitutive activation of NF- κ B to ER-negative phenotype, poor differentiation status, metastasis, and poor prognosis of breast cancers.

Possible mechanisms for constitutive activation of NF- κ B. It is intriguing that the expression of p37(RelA) is observed in all three of the ER-negative cell lines and that only the expression of p37(RelA) correlates with the constitutive NF- κ B activity. Thus, it is important to establish the identity of this protein as

well as its role in NF- κ B activation. There are two possible mechanisms by which p37(RelA) is generated; alternative splicing of RNA and cell-type-specific proteolytic processing of RelA protein. At least three alternatively spliced RelA transcripts have been described, and additional splice variants have been predicted based on sequences in intron 5 and intron 6 of the *relA* gene (12, 35, 36, 42, 47). Although none of the cloned isoforms code for a 37-kDa protein, alternative splicing involving introns 5 and 6 with a change in open reading frame can generate transcripts for a 37-kDa protein (12). Although ER-negative cell-type-specific RelA-encoding transcripts were not detected in Northern analysis, sensitive assays such as RNase protection and reverse transcription-PCR may reveal p37 (RelA)-encoding transcripts (data not shown). At present, there is no evidence for cell-type-specific proteolytic processing of RelA protein. Cell-type-specific proteolytic processing of p100 has been described recently (13), and the possibility of similar processing of RelA cannot be ruled out.

In addition to p37(RelA), overall levels of I κ B α and I κ B β may also determine the constitutive activity of NF- κ B in breast cancer cell lines. Consistent with this possibility, overexpression of either I κ B α or I κ B β reduces the constitutive activity of the NF- κ B/CAT reporter gene in the MDA-MB-231 cell line (Fig. 4E). Thus, reduced expression of either I κ B α or I κ B β is sufficient for constitutive activation of NF- κ B. Apart from a role in constitutive activation, the significance of reduced I κ B β expression in the MDA-MB-231 cell line and I κ B α in the MDA-MB-435 cell line remains to be investigated (Fig. 3B). These factors may determine the duration of inducible NF- κ B activation, as I κ B α and I κ B β mediate transient and persistent activation of NF- κ B, respectively (59). For example, in MDA-MB-231, inducible activation of NF- κ B may be for a short period of time, as this cell line contains very little I κ B β to function as a chaperone that can block association of NF- κ B with newly synthesized I κ B α (57). In contrast, in MDA-MB-435, inducible activation of NF- κ B should be prolonged, as this cell line may not express sufficient I κ B α to terminate NF- κ B activation. In support of this possibility, we observed that TNF- α -inducible DNA binding of NF- κ B after 12 h of incubation was much higher in MDA-MB-435 than in MDA-MB-231 (data not shown). We have also observed that TNF- α -inducible activity of NF- κ B/CAT was much higher in MDA-MB-435 than in MDA-MB-231 (data not shown).

The molecular mechanism responsible for differential I κ B expression in different breast cancer cells is not known. Incubation of MDA-MB-231 and MDA-MB-435 with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a proteasome inhibitor (24), had no effect on the I κ B level, suggesting that rapid turnover of the protein is not responsible for the reduced I κ B protein level (data not shown). Since a mouse I κ B β probe hybridized poorly to human I κ B β RNA in Northern blots and hybridization at a lower stringency resulted in cross-hybridization to 28S and 18S RNAs, cell-type-specific regulation of I κ B β gene expression could not be investigated (data not shown). At present, there is no evidence to suggest a direct role for ligand-activated ER in regulation of p37(RelA) or any known I κ Bs. However, we cannot rule out the possibility that a ligand-independent activation/repression function of ER is responsible for suppression of p37(RelA) and optimum expression of I κ B α and I κ B β in MCF-7 and T47-D. We are currently investigating this possibility by introducing the ER gene into MDA-MB-231 and analyzing I κ B and p37(RelA) expression. Also, it is important to note that there may be additional I κ B proteins whose expression is directly regulated by ER, and lack of these ER-regulated I κ B proteins may be responsible for constitutive NF- κ B activity in ER-negative cell

lines. At the time of preparation of this report, Baeuerle and Baltimore (5) described a new I κ B, I κ B- ϵ , which associates exclusively with RelA and c-Rel.

Although I κ Bs and possibly p37(RelA) determine the extent of constitutive DNA binding activity of NF- κ B, the ER expression level ultimately determines the ability of DNA binding-competent NF- κ B to activate the target genes (Fig. 5). ER may inhibit NF- κ B activity through (i) inhibiting DNA binding of NF- κ B by physically associating with RelA and p50, as shown previously (46, 56), or (ii) increasing the level of the progesterone receptor, which inhibits NF- κ B DNA binding by physically associating with RelA (29, 30). Moreover, NF- κ B-dependent promoter activity has been shown to be enhanced by the *neu* oncogene, which is often overexpressed in ER-negative breast cancers (18, 43). In ER-positive breast cancers, the *neu* gene is negatively regulated by ER (48), and this inhibition of *neu* expression by ER may be responsible for the decreased transactivation potential of NF- κ B even when the DNA binding of NF- κ B is induced. In summation, ER inhibits NF- κ B activity by multiple mechanisms, and it will be interesting to determine which of these mechanisms can be restored by re-introducing ER to ER-negative cells.

Implications of constitutive NF- κ B activation. It is fairly well accepted that the presence of ER identifies those breast cancer patients with a lower risk of relapse and better survival (9). Recent results from studies using clinical material and with antiestrogen-resistant human breast cancer cell lines are consistent with the hypothesis that impaired ER function either due to lack of expression or due to expression of receptor variants is responsible for the development of antiestrogen-resistant tumors which are usually highly invasive, metastatic, and resistant to chemotherapy and radiation treatment (10, 34, 37, 38). Based on the results presented here, we suggest that the growth properties of ER-negative breast cancer cells are due in part to constitutively active NF- κ B. This conclusion is also based on five recent reports indicating an essential role for NF- κ B in protecting cells from TNF- α -, ionizing radiation-, and chemotherapy-induced cell death (6, 33, 62, 64, 66). In addition, the constitutively active NF- κ B may be responsible for clonal selection and outgrowth of ER-negative breast cancer cells, as TNF- α secreted by tumor-infiltrating macrophages and chemotherapeutic agents may selectively kill ER-positive tumor cells. Consistent with this possibility, MDA-MB-231, MDA-MB-435, and RM22-F but not MCF-7, T47-D, and RM22-E form metastatic tumors in nude mice (39, 45). In this respect, tumor cells coinjected with antisense RelA oligonucleotides grow poorly in nude mice (25). Additional studies using MDA-MB-231 and MDA-MB-435 modified to express super-I κ B (64), which can block NF- κ B activation, are necessary to prove the role of NF- κ B in metastatic growth of breast cancer cells in nude mice.

The cellular targets of constitutively active NF- κ B need to be investigated. We believe that the genes involved in apoptosis, proliferation, and metastasis are the major targets, as evidence for the role of NF- κ B in all three cellular events is available. Expression of the uPA gene, which plays a major role in metastasis, may be elevated in ER-negative tumors that contain constitutively active NF- κ B. It is interesting that increased uPA expression has been observed in patients with aggressive breast cancers (14, 15). Moreover, in carcinogen-induced breast cancers of rats, increased expression of uPA has been linked with the estrogen-insensitive stage of the disease (67). In support of this possibility, MDA-MB-231, which displayed maximum NF- κ B DNA binding activity (Fig. 1A) and invades basement membranes much more efficiently than any

other cell lines used in the study (54), expressed a very high level of uPA (data not shown).

Apart from directly influencing the transcription of genes, NF- κ B may indirectly increase the expression of other genes involved in cell proliferation, invasion, and metastasis. For example, RelA has been shown to associate with serum response factor and increase the expression of immediate-early genes involved in cell proliferation in response to growth factors and mitogens (17). NF- κ B may also increase the expression of MMPs such as collagenase I and stromelysin I by physically associating with the transcription factor AP-1 (17, 55, 65). Further experiments exploring the consequences of overexpressing of RelA in ER-positive cells and I κ Bs in ER-negative cells are required to identify the genes whose expression is specifically affected as a consequence of constitutive activation of NF- κ B. Since a number of drugs that inhibit NF- κ B are already in clinical trials for diseases such as AIDS, it will also be interesting to study the effects of NF- κ B inhibitors in combination with ionization radiation and chemotherapeutic drugs on growth, invasion, metastasis, and apoptosis of ER-negative breast cancers.

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