Aberrant Nuclear Factor-κB/Rel Expression and the Pathogenesis of Breast Cancer

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Abstract

Expression of nuclear factor-κB (NF-κB)/Rel transcription factors has recently been found to promote cell survival, inhibiting the induction of apoptosis. In most cells other than B lymphocytes, NF-κB/Rel is inactive, sequestered in the cytoplasm. For example, nuclear extracts from two human untransformed breast epithelial cell lines expressed only very low levels of NF-κB. Unexpectedly, nuclear extracts from two human breast tumor cell lines displayed significant levels of NF-κB/Rel. Direct inhibition of this NF-κB/Rel activity in breast cancer cells induced apoptosis. High levels of NF-κB/Rel binding were also observed in carcinogen-induced primary rat mammary tumors, whereas only expectedly low levels were seen in normal rat mammary glands. Furthermore, multiple human breast cancer specimens contained significant levels of nuclear NF-κB/Rel subunits. Thus, aberrant nuclear expression of NF-κB/Rel is associated with breast cancer. Given the role of NF-κB/Rel factors in cell survival, this aberrant activity may play a role in tumor progression, and represents a possible therapeutic target in the treatment of these tumors. (*J. Clin. Invest.* 1997. 100:2952–2960.) Key words: apoptosis • aromatic hydrocarbons • 7,12-dimethylbenz(*a*)anthracene • rat model • transcription factors

Introduction

The incidence of breast cancer has been steadily increasing over the past 50 yr, and is now one of the leading causes of death among American women between the ages of 40–55 (1). In an attempt to find the causes of this increased incidence, both genetic and environmental factors are being studied. Attention has recently focused on the mechanism by which increased exposure to and bioaccumulation of pollutants might have an etiologic role in breast cancer (2–5). The polycyclic aromatic hydrocarbons (PAHs)[1] such as 7,12-dimethylbenz(*a*)anthracene (DMBA) are specifically of interest with respect to breast cancer (4). The most proximal event in PAH tumorigenesis is the binding of the chemicals to a cytosolic aromatic hydrocarbon receptor (AhR) (6–8). The receptor-ligand complex is translocated to the nucleus where it can bind to and alter the transcriptional activity of DNA that has AhR-responsive elements. One battery of enzymes whose transcriptional induction is a hallmark of DMBA and other PAH exposure is the phase I cytochrome P450 enzymes (9–12). These enzymes aid in the oxidative metabolism of both endogenous substances such as steroids, as well as in the breakdown of exogenous substances such as drugs, chemical carcinogens, and environmental pollutants. The products formed by this oxidative metabolism are often reactive oxygen intermediates. The potential for increased levels of oxidative stress within the cell resultant from exposure to environmental carcinogens led us to hypothesize that this might activate expression of the nuclear factor (NF)-κB/Rel family of transcription factors.

NF-κB/Rel is a family of dimeric transcription factors whose DNA-binding domains have considerable homology with an ~ 300 amino acid region of the v-Rel oncoprotein and was thus termed the Rel homology domain (RHD) (13–15). Classical NF-κB is a heterodimer composed of a 50-kD (p50) and a 65-kD (p65 or RelA) subunit (16). Other members of the mammalian Rel family include c-Rel, p52, and RelB (16–19). The activity of many of these factors is controlled posttranslationally by their subcellular localization. In most cells other than mature B lymphocytes, NF-κB/Rel proteins are sequestered as inactive forms in the cytoplasm by association with inhibitory proteins, termed IκB's, for which IκB-α represents the prototype (16, 20–22). Activation involves IκB degradation, and nuclear translocation of the NF-κB/Rel protein. Many signals that activate NF-κB/Rel do so through a final common pathway of increasing cellular oxidative stress (23). Genes regulated by NF-κB/Rel include those involved in immune and inflammatory responses, cellular proliferation, and adhesion molecules (16, 20, 21). More recently NF-κB/Rel has been implicated in control of apoptosis. For example, we have shown that inhibition of constitutive expression of NF-κB/Rel in B cell lymphomas leads to the induction of apoptosis, and that ectopic c-Rel expression promotes cell survival (24, 25). Recently we have obtained similar data with two untransformed hepatocyte cell lines, which express classical NF-κB

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1. *Abbreviations used in this paper:* AhR, Aromatic hydrocarbon receptor; BSAP; B cell specific activator protein; CAT, chloramphenicol acetyl transferase; dm, double mutant; DMBA, 7,12-dimethylbenz(*a*)anthracene; GST, glutathione-S-transferase; IκB-α, NF-κB/Rel inhibitor-α; NF-κB, nuclear factor-κB; PAH, polycyclic aromatic hydrocarbon; URE, upstream regulatory element.

constitutively (26, 27). Activation of NF-κB/Rel by TNF-α has recently been linked to protection of multiple types of cells from apoptosis (28–31). Here we report that NF-κB/Rel is aberrantly activated in human breast cancer and in rat mammary tumors from the aromatic hydrocarbon-induced model of breast cancer. Inhibition of this activity in human breast cancer cell lines leads to apoptosis. These results suggest an important role for NF-κB/Rel in the pathogenesis of breast cancer and in potential treatment modalities.

Methods

Cell lines. The MCF7 cell line, the prototype of estrogen-dependent breast cancer cells, was established from the pleural effusion of a patient with metastatic adenocarcinoma (32). The 578T tumor cell line was established from a patient with infiltrating ductal carcinoma and does not express estrogen receptors (33); normal breast tissue from this same patient was taken to establish the untransformed breast cell line 578Bst (33). The MCF 10F cell line was established from mammary tissue from a patient with fibrocystic breast disease and is also estrogen receptor negative (34, 35).

Electrophoretic mobility shift analysis. Nuclear extracts for electrophoretic mobility shift analyses (EMSA) were prepared by the method of Dignam et al. (36). URE (5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′; core sequence is underlined) (37), and PU.1 (GATCTACTTCTGCTTTTG, where the core element is underlined) oligonucleotides were end labeled with large Klenow fragment of DNA polymerase and [^{32}P]dNTPs. The electrophoretic mobility shift assay was performed using ~ 2 ng of labeled oligonucleotide (20,000 dpm), 5 μg of nuclear extract, 5 μl of sample buffer (10 mM Hepes, 4 mM dithiothreitol, 0.5% Triton X-100, and 2.5% glycerol), 2.5 μg poly dI-dC as nonspecific competitor and adjusted to 100 mM with KCl in a final volume of 25 μl. This mixture was incubated at room temperature for 30 min. Complexes were resolved in a 4.5% polyacrylamide gel using 0.5× TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0). Specificity of binding was tested using competition analyses in which 10-fold molar excess of wild-type or double mutant URE (URE 1, see below) was added to a binding reaction. For IκB-α blocking experiments, 1 μg of IκB-α-GST fusion protein was added to the reaction after the 30-min incubation, and the mixture allowed to incubate for an additional 1–2 h at 4°C.

Microinjection analysis. Exponentially growing 578T cells were plated on tissue culture dishes. After 24 h, the medium was supplemented with 20 mM Hepes (pH 7.3) to maintain pH when exposed to open air. All cell nuclei in a defined grid were microinjected at 1.4 psi at a rate of ~ 20 cells per minute as described previously (38). Successful microinjection was estimated to occur > 90% of the time. Purified IκB-α-glutathione-S-transferase (IκB-α-GST), kindly provided by U. Siebenlist, or GST protein was used at 1 μg/μl. Affinity purified antibodies to p65 or c-Rel, SC109 and SC070, respectively (Santa Cruz Biotechnology; Santa Cruz, CA), were used at 4 μg/μl in the absence or presence of 4 μg/μl cognate peptide. Double-stranded oligonucleotides were microinjected at a concentration of 200 ng/μl. Oligonucleotides used were: NF-κB, wild-type element from the immunoglobulin κ light chain enhancer (39); wt URE, see above; UREm1 (5′-GATCCAAGTCCG**CC**TTTTCCCCAACC-3′) and UREm2 (5′-GATCCAAGTCCGGGTT**GG**CCCCAACC-3′), mutant forms of the URE κB element (mutated bases are indicated in bold), which fail to bind NF-κB factors.

Cells were stained with 10 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO), 0.1% Triton X-100, 50 μg/ml RNase A in PBS for 15 min and visualized on a Nikon Optiphot Fluorescence microscope (Nikon Inc., Tokyo, Japan) and fluorescent images recorded at 200× using Kodak Tmax 3200 film (Eastman Kodak Co., Rochester, NY). For trypan blue analysis, cloning rings were placed over the microinjected areas. At various time points after microinjection, the supernatant, containing cells that lost adherence during incubation, were transferred to a 96-well plate for trypan blue analysis. The adherent cells were removed by trypsinization and added to the same well. Trypan blue was added to 0.04% and the plate incubated at 37°C. After 30 min, the percentage of positive staining cells was determined by microscopic visualization at a magnification of 100 under brightfield illumination.

Transfection analysis. 578T and MCF7 cells were transfected using the modified calcium phosphate procedure of Chen and Okayama (40) as we have described previously (41). The total amount of plasmid DNA transfected into the cells was adjusted to 25 μg using either pBlueScript or pUC19 plasmid DNA where necessary. Results were normalized to a TK-luciferase construct and are presented as percent of URE$_2$-TK-CAT or p1.6 Bgl-CAT wt activity (mean±SD), which have been set at 100%. Data shown are representative of three independent experiments.

Rat mammary gland and tumor analysis. Virgin female Sprague-Dawley rats fed AIN76 diet were treated according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. Animals were given a single intragastric dose of 15 mg/kg DMBA at 8 wk of age. Tumors, which were first detected by palpation after 7 wk, were rapidly removed at necropsy after death by CO_2 inhalation. Normal mammary glands, similarly excised from untreated control rats, and tumors were frozen in liquid nitrogen. Animals were killed 16 wk after DMBA treatment at 24 wk of age, except when tumors appeared ulcerated. Samples were pulverized on dry ice using a Bessman tissue pulverizer (Spectrum Industries, Gardena, CA). Frozen tissue powder was homogenized (0.5 grams/ml) in TEGT/MO buffer [50 mM Tris/HCl, 1 mM EDTA, 10% (vol/vol) glycerol, 10 mM monothioglycerol, 10 mM sodium molybdate, pH 7.4 containing 0.02% sodium azide] using a Polytron. After the initial burst proteolytic inhibitors were added to a final concentration of: 0.5 mM PMSF, 1 μg/ml leupeptin, 100 μg/ml aprotinin, 10 μg/ml pepstatin, and 100 μg/ml bacitracin. Homogenates were centrifuged for 10 min at 3,000 rpm at 2°C. Nuclear pellets were extracted via the procedure of Dignam et al. (36) and analyzed as described above.

Human breast cancer specimen analysis. Primary human breast cancer tissue, obtained with IRB approval, was frozen in dry ice and stored frozen until samples were processed for nuclear protein extraction. Nuclear pellets were obtained as described above and washed twice with TEGT/MO buffer and proteins extracted in TEGT/MO buffer plus protease inhibitors adjusted to 0.4 M KCl for 45 min. The debris was removed by centrifugation at 30,000 rpm and the nuclear extract stored frozen. For immunoblot analysis, 20–100 μg of protein were resolved on a 10% polyacrylamide gel and transferred to a 0.45-μm pore PVDF membrane (Millipore). Blots were blocked for 1 h at room temperature in 5% milk (Carnation) in Tris-buffered saline (TBS), then probed with antibody diluted (1:1,000) in 5% milk in TBS for 1 h at room temperature. After three washes in 0.5% NP-40 in TBS, blots were incubated in secondary horseradish peroxidase (HRP)-conjugated goat anti–rabbit antibody at 1:1,000 dilution for 1 h at room temperature. Bands were visualized by chemiluminescence.

Results

Human breast epithelial tumor cell lines constitutively express high levels of functional nuclear NF-κB/Rel activity. To begin to analyze the role of NF-κB/Rel in regulation of epithelial cell proliferation, we performed EMSA on nuclear extracts of untreated, exponentially growing breast tumor and untransformed breast epithelial cell lines to assess basal NF-κB/Rel binding activity. The oligonucleotide containing the NF-κB URE from the c-*myc* gene (37) was used as probe; this oligonucleotide has been shown to bind multiple NF-κB/Rel complexes efficiently (41–44). Significant levels of nuclear NF-κB/Rel complexes were detected in MCF7 (32) and 578T (33) hu-

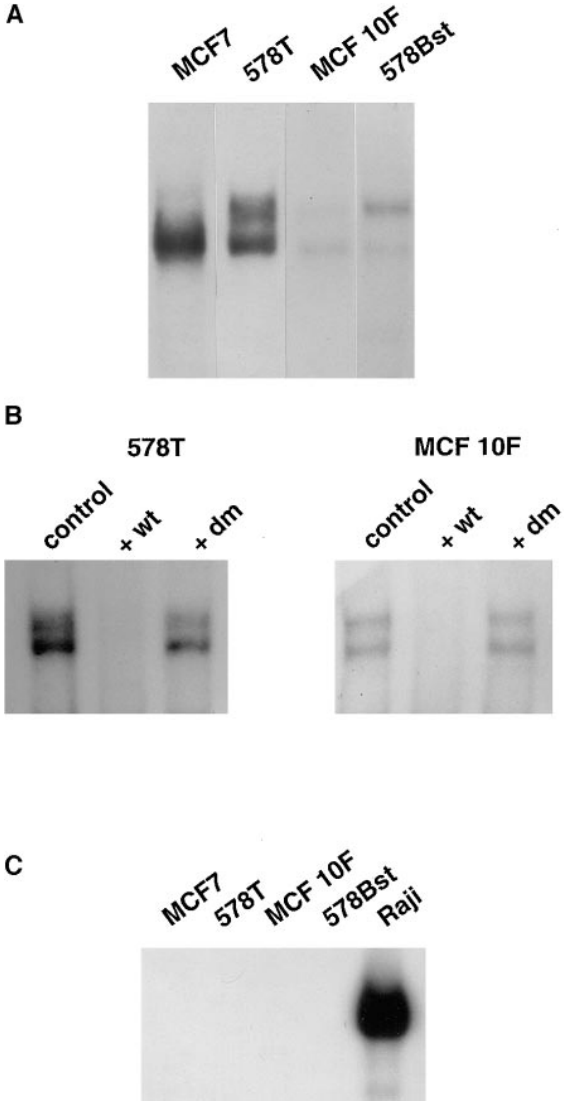


Figure 1. Transformed human breast cancer cell lines constitutively display high levels of nuclear NF-κB/Rel factor binding. (*A*) Nuclear extracts from transformed MCF7 and 578T and untransformed MCF 10F and 578Bst breast epithelial cell lines in exponential growth were used in EMSA analysis with URE NF-κB element oligonucleotide as probe. The level of binding varied amongst the several MCF7 isolates studied, which is in accordance with the recent inability of another group to detect significant levels in their nuclear preparations (70). (*B*) Nuclear extracts from 578T and MCF 10F cells were used in EMSA analysis with the URE NF-κB oligonucleotide as probe in the absence (*control*) or presence of 10-fold molar excess unlabeled wild-type (*wt*) or double mutant URE m1 (*dm*) as competitor. (*C*) EMSA of PU.1 binding was performed, as above. Nuclear extract from the Raji Burkitt lymphoma B cell line was used as a positive control.

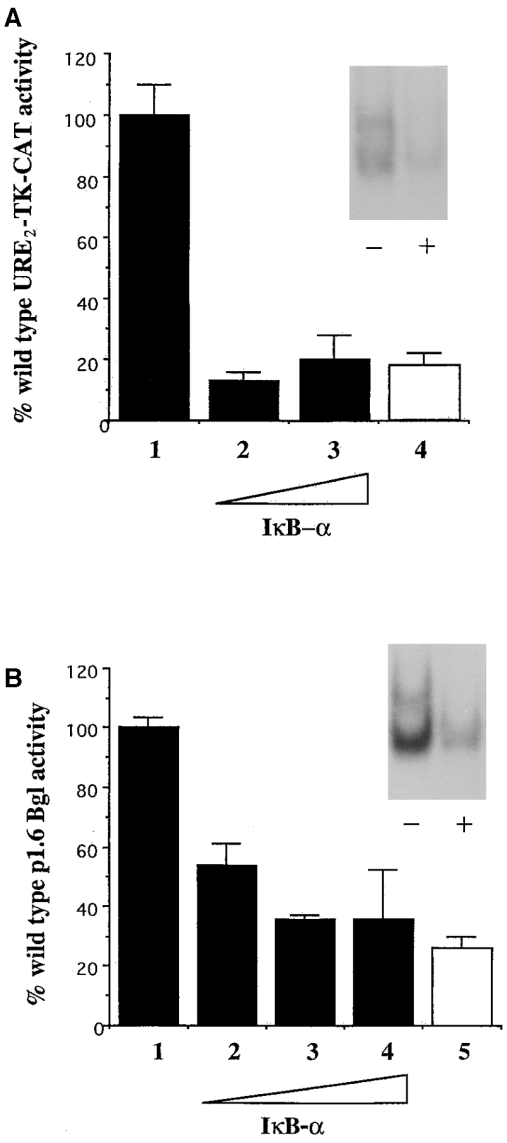


Figure 2. Constitutive nuclear NF-κB/Rel in transformed breast cancer lines is functional. (*A*) 578T cells were transfected in duplicate with either 10 μg of the URE_2-TK-CAT wt reporter plasmid in the absence (bar *1*) or presence of increasing amounts of IκB-α expression vector (3 and 9 μg, bars *2* and *3*, respectively), or with 10 μg of the URE_2-TK-CAT dm plasmid (bar *4*). The total amount of DNA transfected into the cells was adjusted to 25 μg using pBlueScript plasmid DNA. Results were normalized to a TK-luciferase construct and are presented as percentage of URE_2-TK-CAT wt activity (mean±SD), which is set at 100%. (*Inset*) Nuclear extracts from exponentially growing 578T cells were incubated in the absence (−) or presence (+) of 1 μg of IκB-α-GST protein and subjected to EMSA analysis using the URE oligonucleotide as probe. (*B*) MCF7 cells were transfected in duplicate with 13 μg of p1.6 Bgl-CAT wt plasmid in the absence (bar *1*) or presence of 3, 6, or 12 μg of IκB-α expression vector (bars *2*, *3*, or *4*, respectively). Additionally cells were transfected with 13 μg of p1.6 Bgl-CAT dm plasmid containing two G to C conversions in its two identified NF-κB elements (46) (bar *5*). The total amounts of DNA transfected into cells were equalized to 25 μg using pUC19 DNA. The data are presented relative to p1.6 Bgl-CAT wt activity (mean±SD), which is set at 100%. (*Inset*) Nuclear extracts from exponentially growing MCF7 cells were incubated in the absence (−) or presence (+) of 1 μg of IκB-α-GST protein and subjected to EMSA analysis using the URE oligonucleotide as probe.

man breast tumor cell lines, but not in untransformed MCF 10F (34, 35) and 578Bst (33) breast epithelial cell lines (Fig. 1 *A*). This finding was unexpected since nuclear NF-κB/Rel is thought to be restricted primarily to B lymphocytes (16, 21, 39). Nuclear extracts from MCF7 cells displayed several complexes that resolved as a broad lower band and a faint upper band. The ratio and intensity of these two bands varied with different isolates of this line (compare Fig. 1 *A* and Fig. 2 *B*,

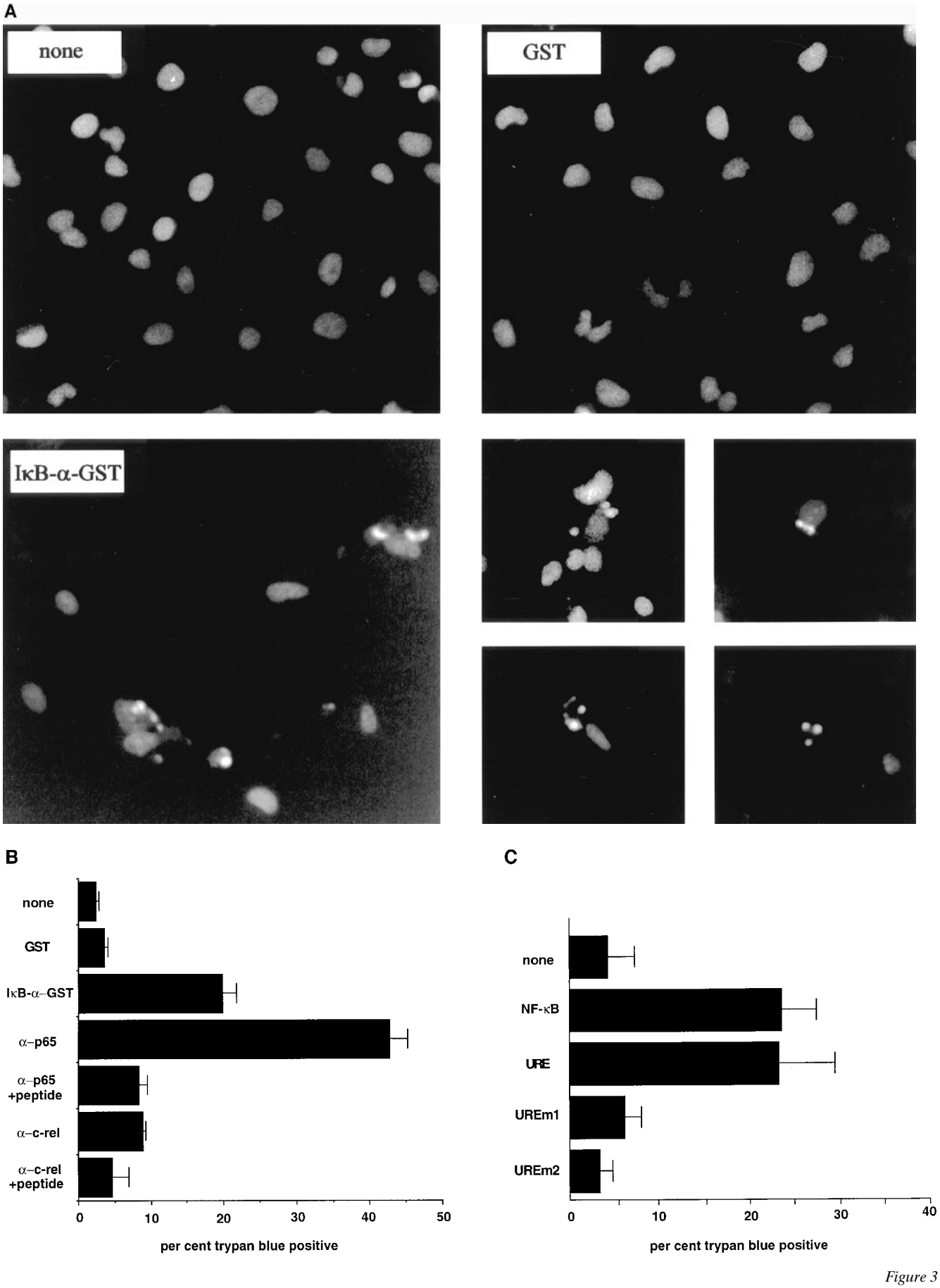
and data not shown), which is known to change phenotype in culture. Multiple complexes were also seen with nuclear extracts from 578T cells. Nuclear extracts from the untransformed MCF 10F and 578Bst cell lines displayed only very low levels of NF-κB/Rel binding, as expected. The bands observed with all cell lines tested comigrated with complexes obtained using nuclear extracts from WEHI 231 B lymphoma cells (data not shown), a cell line known to express high levels of NF-κB/Rel (37, 39, 44). Binding specificity of these proteins to URE was confirmed by successful competition with 10-fold excess unlabeled wild-type oligonucleotide, whereas excess unlabeled mutant oligonucleotide essentially had no effect on binding (Fig. 1 *B* and data not shown).

Transient transfection analyses were performed to test functional Rel activity in the tumor lines. The URE_2-TK-CAT wild-type (wt) and URE_2-TK-CAT double mutant (dm) constructs have two copies of the URE NF-κB binding element from the c-*myc* gene in either wild-type or mutant form, respectively, linked to the heterologous thymidine kinase (TK) promoter and the chloramphenicol acetyltransferase reporter gene (CAT) (37). Cultures of 578T cells at 70% confluence were transiently transfected in duplicate (Fig. 2 *A*). The wild-type element vector gave approximately sevenfold higher levels of activity than the double mutant; an average of 9±2-fold higher levels of activity were obtained in six experiments. A similar value of 11±3-fold ($n = 6$) was obtained using MCF7 cells. To further confirm the observed transactivation was indeed due to constitutive NF-κB/Rel activity, cells were cotransfected in duplicate with URE_2-TK-CAT wt and increasing amounts of an IκB-α expression vector (45). A decrease in relative CAT activity of the wild-type construct to that of the double mutant vector was observed upon cotransfection of as little as 3 μg of the IκB-α vector, indicating that the observed activity of the URE_2-TK-CAT wt construct was due to constitutively functional NF-κB/Rel in the cells (Fig. 2 *A*). Similarly, cotransfection of MCF7 cells was performed with a natural promoter construct responsive to NF-κB, the c-*myc* promoter/exon1 p1.6 Bgl-CAT construct (46). Increasing concentrations of the IκB-α expression vector brought the activity of the wild-type construct down to that of the construct with mutated NF-κB elements (Fig. 2 *B*). Consistent with the effects of the inhibitor in transient transfection, addition of IκB-α-GST protein to the binding reactions with nuclear extracts from either transformed cell line abrogated formation of the upper complexes and significantly reduced the level of the more rapidly migrating complex (*insets*, Figs. 2, *A* and *B*). This finding is expected as IκB-α protein has been shown to prevent binding via selective interaction with p65, c-Rel, and RelB (present in slower migrating complexes); inhibition of binding of p50 or p52 appears to require somewhat higher concentrations of inhibitor protein (reviewed in 16). Lastly, the specific activation of NF-κB/Rel binding as opposed to a general elevation in B cell transcription factors was confirmed by EMSA for the Ets transcription factor member PU.1, expressed predominantly in B lymphocytes and myeloid cells (47–49). No PU.1 binding was observed with nuclear extracts from the breast epithelial cell lines, whereas binding of nuclear extracts from the Burkitt lymphoma Raji B cells to the PU.1 oligonucleotide was easily detected (Fig. 1 *C*). Similarly, we failed to detect binding of the B cell–specific activator protein (BSAP) with nuclear extracts from the untransformed or transformed breast epithelial lines (50) (data not shown). Thus, MCF7 and 578T breast cancer cells have selectively activated constitutive nuclear NF-κB/Rel factors.

Inhibition of the constitutive NF-κB/Rel activity in 578T cells induces apoptosis. Recently, we demonstrated that NF-κB/Rel rescues immature B lymphoma cells and hepatocytes from undergoing apoptosis (24–27) and a similar role in cell survival has been demonstrated upon its induction after TNF-α treatment of various cell lines (28–31). If the aberrant NF-κB/Rel expression we detect in breast cancer cells plays a similar role, specific inhibition of its activity should result in cell death. Thus, we used a microinjection strategy to selectively inhibit NF-κB/Rel expression in breast cancer cells. Areas of ~ 4 mm^2 were defined and all 578T breast cancer cells within the grid were microinjected with either the specific NF-κB/Rel inhibitory IκB-α-GST fusion protein or GST protein alone as control. After 3.5 h, the nuclear morphology of the cells was examined by microscopic observation after staining with propidium iodide. Nuclear condensation, a hallmark of apoptosis, was clearly visualized in a field of cells microinjected with IκB-α-GST protein (Fig. 3 *A*, *left bottom panel*), but not with GST protein or in noninjected cells (Fig. 3 *A*, *top panels*). Typical cells with condensed chromatin after microinjection of IκB-α-GST are shown in Fig. 3 *A* (*right bottom panel*). A marked drop in cell density with time was noted after microinjection with IκB-α-GST protein but not control GST protein, possibly reflective of the observation that cells undergoing apoptosis detach and float off the dish surface.

To obtain more quantitative analysis, trypan blue staining was used as a measure of cell viability after microinjection with IκB-α-GST or GST protein, as above, or with affinity purified antibodies to either the p65 or c-Rel subunit. We noted that the adherent cell density was reduced by $\sim$ 50–60% after injection with IκB-α-GST (Fig. 3 *A*), whereas only 4–5% of the cell population injected with GST protein alone lost adherence (data not shown). Therefore, nonadherent and adherent cells were combined and analyzed for loss of cell viability 20–22 h after microinjection (Fig. 3 *B*). 20% of the cells microinjected with IκB-α-GST stained positive for trypan blue, compared to $<$ 3% of cells microinjected with GST or nonmicroinjected cells. Thus, IκB-α–mediated inhibition of NF-κB/Rel caused death of 578T breast cancer cells by apoptosis. In a preliminary supershift EMSA analysis, 578T cells were found to express significant levels of p65 whereas only low levels of c-Rel subunits were detected (data not shown). Microinjection with an affinity purified antibody to p65 induced cell death in $\sim$ 45% of 578T cells within 20–22 h, which was inhibited by preincubation of the antibody with 40-fold molar excess of its cognate peptide (Fig. 3 *B*). In contrast, microinjection of the antibody against c-Rel induced only modest additional loss of cell viability over background levels seen with microinjection with GST protein alone.

To further evaluate the effects of specific inhibition of NF-κB/Rel activity, we also monitored loss of cell viability after microinjection of double-stranded (ds) oligonucleotides harboring either wild-type or mutated NF-κB elements. Recent studies have demonstrated that microinjection of ds oligonucleotides containing a transcription factor binding site can compete in vivo and inhibit the activity of the factor (51, 52). Microinjection of oligonucleotides containing the wild-type element from the κ light chain enhancer (κB) or from the c-*myc* gene (URE) induced significant levels of apoptosis within 20 h (Fig. 3 *C*). In contrast, microinjection of oligonucleotides con-


A
none
GST
IκB-α-GST
B
none
GST
IκB-α–GST
α–p65
α–p65
+peptide
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+peptide
0
10
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40
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per cent trypan blue positive
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Figure 3

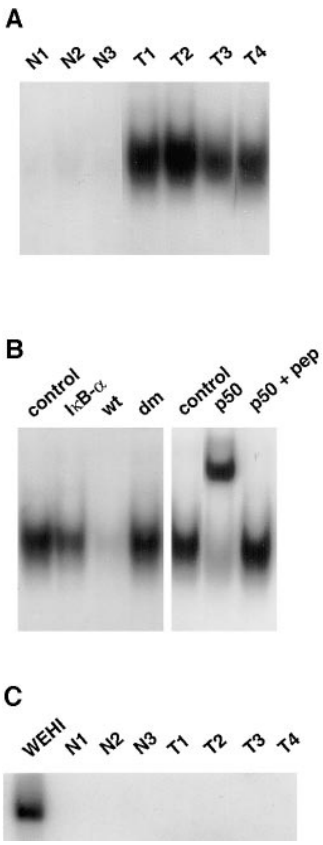

Figure 4. Nuclear expression of NF-κB/Rel in primary rat mammary tumors but not normal mammary gland. (*A*) Nuclear extracts were prepared from mammary glands from three untreated control rats (*N1–N3*) or from mammary tumors from DMBA-treated rats (*T1–T4*) and equal amounts (5 μg) subjected to EMSA for binding to the URE oligonucleotide, as described above. (*B*) EMSA was performed with a nuclear extract from tumor T1 in the absence (*control*) or presence of 1 μg IκB-α-GST protein (IκBα), 50× molar excess unlabeled oligonucleotide containing URE wild type (*wt*) or mutant (*dm*) sequences (*left*). Alternatively, the antibody to the p50 subunit in the absence (*p50*) or presence of cognate peptide (*p50 + pep*) was added to the binding reaction. (*C*) EMSA was performed with nuclear extracts described in part A with the PU.1 oligonucleotide. WEHI 231 B cell nuclear extracts were used as a positive control.

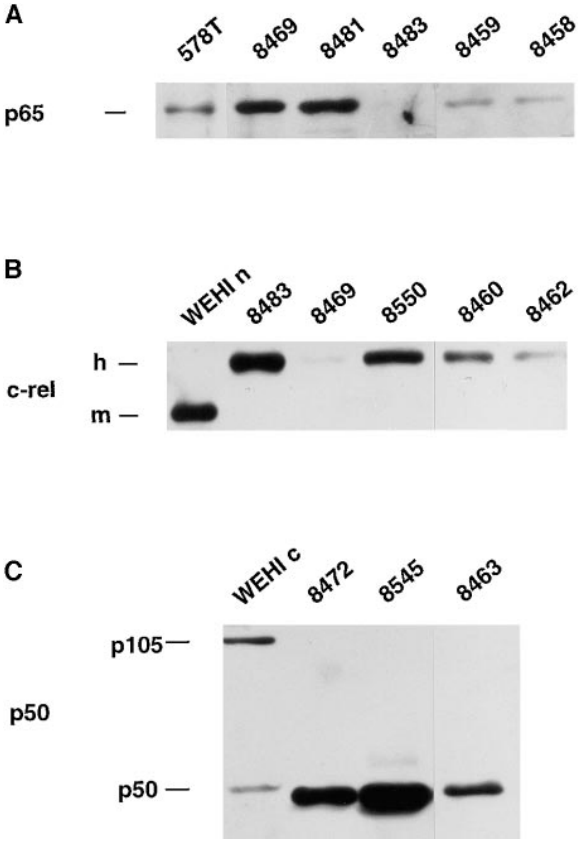

Figure 5. Nuclear expression of NF-κB/Rel subunits in primary human breast cancer tissue. (*A*) p65: Samples of nuclear extracts from multiple tumor specimens (100 μg/lane) were analyzed using the anti-p65 antibody #1226. (*B*) c-Rel: immunoblot analysis was performed on nuclear extracts (50 μg/sample) using an antibody against c-Rel protein (SC070; Santa Cruz Biotechnology Co.). (*C*) p50: immunoblot analysis was performed on nuclear extracts (50 μg/sample) using an antibody against p50 protein (SC114; Santa Cruz Biotechnology Co.). Patient samples are indicated by four digit numbers. As controls for detection, a nuclear extract from exponentially growing 578T cells (20 μg) was used in panel A, and nuclear (*n*) or cytoplasmic (*c*) extracts (50 μg) from WEHI 231 murine B cells (*WEHI*) were used in panels *B* and *C*, respectively. *h*, Human; *m*, mouse.

taining two mutations of the URE (UREm1 and UREm2) that prevent binding of NF-κB/Rel factor did not significantly increase cell death above background levels seen with uninjected cells. In summary, selective inhibition of NF-κB/Rel activity by these three different strategies resulted in apoptosis of 578T breast cancer cells.

DMBA-induced rat mammary tumors express high levels of nuclear NF-κB/Rel binding activity. To verify that the observed nuclear expression of NF-κB/Rel factor complexes in the breast cancer cell lines was not the result of in vitro cell culturing, we have extended these observations to a widely used in vivo rat model of breast cancer. Treatment of female Sprague-Dawley rats with a single dose of the polycyclic aromatic hydrocarbon DMBA results in the induction of mammary gland (breast) tumors within 7–20 wk (53). These tumors are generally well differentiated and retain their hormonal responsiveness. Nuclear extracts were prepared from multiple DMBA-induced rat mammary tumors and from normal mammary glands from untreated rats as controls. High levels of binding to the URE NF-κB oligonucleotide, essentially comparable to that seen with WEHI 231 B cells, were obtained with nuclear extracts from 86% of the 35 tumor specimens analyzed (Fig. 4 *A*, lanes *T1–T4* and data not shown). In contrast, no significant levels of NF-κB/Rel binding were observed with nuclear extracts from the normal mammary gland, as expected (Fig. 4 *A*, lanes *N1–N3* and data not shown). The specificity of the NF-κB/Rel binding was confirmed by competition with excess unlabeled oligonucleotides containing wild-type but not mutant NF-κB elements and the ability of an antibody against the

Figure 3. Microinjection of 578T cells with IκB-α protein induces apoptosis. (*A*) Propidium iodide staining of 578T cells in exponential growth (*none*), and 3.5 h after microinjection with 1 μg/μl GST protein alone or 1 μg/μl IκB-α-GST fusion protein. All cells shown within these representative fields were microinjected. (The left bottom panel displays a representative region of the full field; however, since the numbers of cells microinjected with IκB-α protein were greatly reduced, individual clusters of cells are shown in the smaller panels.) (*B*) 578T cells (between 76 and 141 cells per sample) were microinjected in duplicate and cells stained with trypan blue 20–22 h after microinjection to assess cell viability. Data are expressed as mean±SD and are representative of two experiments. (*C*) 578T cells (between 84 and 164 cells per sample) were microinjected in duplicate with the indicated double stranded wild-type or mutant NF-κB oligonucleotide, and analyzed for trypan blue staining 20 h after treatment. Data are expressed as mean±SD.

p50 subunit and addition of IκB-α-GST protein to significantly ablate binding activity (Fig. 4 *B*). No binding to the PU.1 oligonucleotide was seen with nuclear extracts from either normal mammary gland or mammary tumors (Fig. 4 *C*). These results indicate that the observed NF-κB/Rel binding is not due to contaminating B cells. Thus, NF-κB/Rel expression is selectively induced in vivo within rat mammary gland tumors but is not present normally in mammary glands, suggesting a significant association between the activation of NF-κB/Rel expression and mammary tumor formation.

Nuclei of primary human breast tumor specimens contain NF-κB/Rel subunits. To determine whether NF-κB/Rel factors are constitutively expressed in nuclei of primary human breast cancer tissue, samples procured after surgical removal of breast tumors were analyzed for potential NF-κB/Rel subunit expression by immunoblotting. Nuclear extracts from 13 patient tumor samples were tested with an antibody against the p65 subunit (#1226 [54], kindly provided by N. Rice, National Cancer Institute, Frederick, MD) (Fig. 5 *A* and data not shown). Eight tumors had detectable levels of a band that comigrated with the p65 subunit derived from 578T cells. A second antibody against p65 (SC372; Santa Cruz Biotechnology) was used with its cognate peptide to confirm the specificity of the positive signal seen with the tumor samples (data not shown). Detection of p65 in the nuclear extracts is not due to contamination with cytoplasmic proteins as nuclear extracts from eight tumors were tested and found negative for the cytoplasmic IκB-α protein (data not shown). Overall, nuclear extracts from 15 of 23 specimens displayed positive staining for p65: 8 of 13 specimens were found positive using the #1226 antibody (and four confirmed with SC372) and 7 of 10 specimens were positive using the SC372 antibody (Table I).

Table I. Immunoblot Analysis of Nuclear NF-κB/Rel Subunit Expression in Human Breast Cancer Tissues

Code*	p65	c-Rel	p50
8445	+	+	+
8446	−	+	+
8448	+	+	+
8450	+	+	+
8458	+	+	+
8459	+	+	+
8460	+	+	+
8462	+	+	−
8463	−	+	+
8469	+	+	+
8470	−	+	+
8471	+	+	+
8472	−	+	+
8479	−	−	NA‡
8481	+	+	−
8483	−	+	+
8484	+	+	+
8487	+	+	+
8488	+	+	+
8545	−	+	+
8547	+	−	+
8549	−	−	NA‡
8550	+	+	+
Total positive	15/23	20/23	19/21

*Patient samples are indicated by four digit numbers. ‡Not available.

Expression of the c-Rel subunit was also assessed by immunoblotting (Fig. 5 *B*). A band that migrated marginally slower than the mouse c-Rel protein was detected with antibody SC070 (Santa Cruz Biotechnology), which was competed away upon addition of cognate peptide (data not shown). Of 23 specimens tested, 20 were found to contain nuclear c-Rel protein (Fig. 5 *B* and data not shown); the level of this expression was in many cases comparable to that seen in WEHI 231 cells, which express very high levels of this subunit (37, 39). In addition, nuclear extracts from tumors contained significant levels of the p50 subunit (Fig. 5 *C* and data not shown), which was shown to be specific by successful competition with cognate peptide (data not shown). Overall, 19 of 21 tumors were positive for this subunit. The absence of detectable p105 precursor, as seen in the cytoplasmic extracts of WEHI 231 B cells (WEHI c), further confirms the absence of cytoplasmic contamination of these nuclear extracts (Fig. 5 *C*). The results, summarized in Table I, indicate that most primary human breast cancer tissue samples express the transactivating p65 and/or c-Rel subunits of NF-κB/Rel. Some samples were negative for both subunits; however, a more complete analysis of breast tumors for all of the NF-κB/Rel subunits may reveal the presence of additional subunits. Preliminary EMSA, which is more sensitive than immunoblotting, has confirmed variable levels of NF-κB/Rel binding in multiple human patient samples (data not shown). Overall, these findings indicate that the nuclear localization of NF-κB/Rel expression is a characteristic of human breast tumor cell lines, DMBA-induced rat mammary tumors, and primary human breast cancer tissue.

Discussion

We have analyzed three different systems of breast cancer: in vitro human breast cancer cell lines, an in vivo rat model of breast cancer, and human tissue samples from primary breast tumors. All three have revealed an association between activated NF-κB/Rel and breast cancer. Direct inhibition of the functional NF-κB/Rel factors expressed in 578T cells by microinjection of IκB-α protein, an antibody to the p65 subunit, or ds oligonucleotides containing NF-κB elements led to induction of death of these cells via apoptosis. This study extends our recent work demonstrating that inhibition of the normal constitutive NF-κB/Rel activity in B cell lymphomas leads to cell death (24, 25), to breast cancer where NF-κB/Rel has been aberrantly activated. Our findings suggest that down-regulation of NF-κB/Rel may be useful in the treatment of this disease. Many antioxidants such as pentoxifylline (55) and *N*-acetyl cysteine (56), which are already in clinical use, have been found to repress NF-κB/Rel activity. Preliminary evidence suggests that these agents have significant inhibitory effects on proliferation of breast cancer cells in culture (unpublished observations). Thus, while the efficacy of a specific agent may depend on the nature of the NF-κB/Rel and IκB subunits expressed, our studies suggest NF-κB/Rel is a novel therapeutic target for the treatment of breast cancer.

There has been a steady increase in the incidence of breast cancer over the last several decades, and it has been proposed that this may reflect an increased exposure to environmental

carcinogens such as DMBA (5, 57, 58). Our study of DMBA-induced tumors in rats shows an association between DMBA exposure, breast tumor formation, and activation of NF-κB/Rel. It has been proposed that carcinogens promote tumor progression through DNA damage. The activation of NF-κB/Rel in the tumors, which would promote cell survival, suggests an additional indirect effect of this carcinogen on tumor progression. The mechanism of NF-κB/Rel activation by DMBA remains to be determined. As discussed above, however, a hallmark of DMBA exposure is an increase in P450 enzyme levels with a resultant increase in oxidative stress, which is known to activate NF-κB/Rel (11, 23, 59).

We have recently reported that ectopic expression of c-Rel in WEHI 231 B cells leads to extensive protection from apoptosis induced by engagement of surface immunoglobulin, treatment with TGF-β1, or addition of the protease inhibitor TPCK (24, 25). Ectopic c-Rel expression plays a similar role in protection of hepatocytes from TGF-β1–induced cell death (26). Furthermore, inhibition of endogenous p50/p65 NF-κB activity within these hepatocytes leads to cell death via apoptosis (27). Thus, the finding that many of the breast cancer specimens express c-Rel and p65 suggests that NF-κB/Rel factors play a similar role as a survival gene in these tumors. The primary target(s) of these factors remain a major question. Candidate genes include the known antiapoptotic genes, Bcl-2 and Bcl-X_L. One additional candidate for target is the c-*myc* gene, which is extensively regulated through its two NF-κB/Rel elements (42, 44, 46). Recently, we have shown that ectopic c-*myc* expression protects against apoptosis of B cells (60). Interestingly, studies on patient material and transgenic mice have implicated overexpression of the c-*myc* gene in the etiology of breast cancer (61–66). In human breast cancer, analysis of tumors shows an association between overexpression of the c-*myc* gene and poor prognosis, especially in node-negative patients (61, 62, 67, 68). These findings raise the possibility that the activation of c-*myc* gene by NF-κB may serve to promote both tumor cell protection from apoptosis as well as neoplastic transformation.

With the expanded use of sensitive mammographic and tissue sample screening procedures, there has been a significant rise in detection of potentially premalignant lesions. The prevalence of benign breast conditions or fibrocystic changes is estimated at $> 85\%$ in women in the U.S. Approximately one-third of U.S. women undergo breast biopsies; the vast majority of these biopsies show benign disease that are not associated with an increased risk of cancer (69). The quest for markers to positively identify which of these lesions will progress to malignancy is critical to help develop strategies for treatment of benign breast disease and to improve early detection of breast cancer. Nuclear NF-κB/Rel expression is associated with neoplastic breast tissue in humans and laboratory rats. Studies are underway to elucidate any association between the stages of neoplastic transformation and expression of NF-κB/Rel factors as markers of disease.

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