roles for NF-κB2/p52 and for Bcl-3

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Members of the NF-κB/Rel transcription factor family have been shown recently to be required for cellular transformation by oncogenic Ras and by other oncoproteins and to suppress transformation-associated apoptosis. Furthermore, NF-κB has been shown to be activated by several oncoproteins including HER2/Neu, a receptor tyrosine kinase often expressed in human breast cancer. Human breast cancer cell lines, human breast tumors and normal adjacent tissue were analysed by gel mobility shift assay, immunoblotting of nuclear extracts and immunohistochemistry for activation of NF-κB. Furthermore, RNA levels for NF-κB-activated genes were analysed in order to determine if NF-κB is functionally active in human breast cancer. Our data indicate that the p65/RelA subunit of NF-κB is activated (i.e., nuclear) in breast cancer cell lines. However, breast tumors exhibit an absence or low level of nuclear p65/RelA but show activated c-Rel, p50 and p52 as compared to nontumorigenic adjacent tissue. Additionally, the IκB homolog Bcl-3, which functions to stimulate transcription with p50 or p52, was also activated in breast tumors. There was no apparent correlation between estrogen receptor status and levels of nuclear NF-κB complexes. Transcripts of NF-κB-regulated genes were found elevated in breast tumors, as compared to adjacent normal tissue, indicating functional NF-κB activity. These data suggest a potential role for a subset of NF-κB and IκB family proteins, particularly NF-κB/p52 and Bcl-3, in human breast cancer. Additionally, the activation of functional NF-κB in these tumors likely involves a signal transduction pathway distinct from that utilized by cytokines. *Oncogene* (2000) **19,** 1123–1131

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Introduction

As with virtually all malignancies, breast cancer is associated with dysregulated expression of growth factors and growth factor receptors as well as kinases associated with growth factor signaling pathways (Dickson and Lippman, 1995; Resnik *et al*., 1998). The ultimate downstream effectors of these pathways are transcription factors. Thus, dysregulated transcription is likely to play an important role in cancer since it has been shown that the transcription factors c-*jun*, ets, c-*myc* and NF-κB are each required for cellular transformation controlled by oncogenic Ras alleles (Johnson *et al*., 1996; Langer *et al*., 1992; Sklar *et al*., 1991; Finco *et al*., 1997). The ultimate gene targets of these transcription factors that contribute to oncogenesis are poorly understood.

NF-κB is a transcription factor that is known to play an important role in controlling immune and inflammatory responses (see Baldwin, 1996; Ghosh *et al*., 1998; Verma *et al*., 1995). There are presently five members of the mammalian NF-κB/Rel family: NF-κB/p50, NF-κB2/p52, c-Rel, RelA/p65 and RelB. The p50 and p52 NF-κB subunits are derived from larger precursor products, p105 and p100 respectively, or from differential translation of their mRNAs. The classic form of NF-κB, the heterodimer of the p50 and 65 subunits, is normally retained in the cytoplasm through interactions with inhibitor proteins IκBα and IκBβ. Inductive stimuli (TNFα, IL-1, bacterial endotoxin, etc.) lead to the phosphorylation and degradation of IκB, allowing NF-κB to enter the nucleus and regulate gene expression (reviewed in Baldwin, 1996; Ghosh *et al*., 1998; Verma *et al*., 1995). IκBα is inducibly phosphorylated on serines 32 and 36 following activation of the IκB kinase complex (Ghosh *et al*., 1998). Following its degradation, IκBα is transcriptionally upregulated due to NF-κB sites in its promoter which leads to a suppression of p65- and c-Rel-containing heterodimers. IκBβ also interacts with the same heterodimers as IκBα but is degraded with slower kinetics and functions in the persistent activation of NF-κB (Ghosh *et al*., 1998). Bcl-3, a noninhibitory member of the IκB family (Ohno *et al*., 1990; Kerr *et al*., 1992) that is associated with certain leukemias and lymphomas (McKeithan *et al*., 1997), functions to stimulate transcription through interactions with the p50 or p52 NF-κB subunits (Bours *et al*., 1993; Fujita *et al*., 1993) and to increase nuclear levels of p50 homodimers (Zhang *et al*., 1994; Watanabe *et al*., 1997). Bcl-3-p50 complexes can be activated following inducible phosphorylation of the p105 precursor by the IκB kinase (Heissmeyer *et al*., 1999). Recently, it was shown that Bcl-3 interacts with transcriptional co-activators, including the Tip60 histone acetyltransferase (Dechend *et al*., 1999). Gene knockout studies confirm the important roles of the NF-κB family in immune function and also indicate that the RelA/p65 subunit is required to suppress liver cell apoptosis (reviewed in Baldwin, 1996; Ghosh *et al*., 1998; Verma *et al*., 1995; and see Beg *et al*., 1995).

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Additionally we, Wang *et al.* (1996), and others (Arsura *et al.*, 1996; Beg and Baltimore, 1996; Liu *et al.*, 1996; Van Antwerp *et al.*, 1996; Chu *et al.*, 1997), have shown that NF-κB suppresses apoptosis initiated by tumor necrosis factor, chemotherapy or radiation.

Evidence for a role of NF-κB in oncogenesis is extensive. The founding member of the NF-κB family, c-Rel, is the cellular homolog of v-Rel, the transforming gene of avian reticuloendotheliosis virus (Gilmore, 1997). One of the NF-κB genes, NF-κB2/p52 (lyt-10), is found translocated in some lymphoid neoplasms (Chang *et al.*, 1995) as is the IκB family member Bcl-3 (McKeithan *et al.*, 1997; Michaux *et al.*, 1997). Furthermore, several oncoproteins, including HER2/Neu, Ras and Bcr-Abl, are known to activate NF-κB and NF-κB is required for transformation induced by Ras and by Bcr-Abl (Galang *et al.*, 1996; Finco *et al.*, 1997; Reuther *et al.*, 1998). Importantly, NF-κB activation suppresses a transformation-induced apoptosis associated with oncogenic Ras expression (Mayo *et al.*, 1997) and expression of the RelA/p65 subunit is required for growth of Hodgkin's lymphoma (Bargou *et al.*, 1997). Consistent with this, IκBα is found mutated in certain cases of Hodgkin's disease (Cabannes *et al.*, 1999). Recently, it was shown that NF-κB transcriptionally regulates the gene encoding cyclin D1 and, correspondingly, promotes cell growth (Hinz *et al.*, 1999; Guttridge *et al.*, 1999).

NF-κB expression has been analysed in human breast cancer cell lines and in breast cancer tissue. Dejardin *et al.* (1995) found that the 100 kD precursor of the NF-κB2/p52 subunit was overexpressed in a majority of breast cancer samples and in cancer cell lines, suggesting a role for the precursor in retaining classic forms of NF-κB in the cytoplasm. Nakshatri *et al.* (1997) found that NF-κB RelA/p65 is activated (i.e., nuclear) in the majority of human breast cancer cell lines and that the relative level of NF-κB was inversely correlated with estrogen receptor (ER) expression. Recently, Sonenshein and colleagues (Sovak *et al.*, 1997) found increased levels of NF-κB in human breast cancer samples and that inhibition of NF-κB in a breast cancer cell line led to apoptosis. We have explored the NF-κB subunit composition in human breast tumors and have found that the p50, p52, and c-Rel subunits are found in the nucleus in virtually all samples of breast cancer. Importantly, the p52 subunit was significantly upregulated in tumor tissue as compared to stromal fibroblasts or adjacent, non-tumor breast epithelium. There was no apparent correlation between ER status and NF-κB activation in the tumor samples. Interestingly, the p65 subunit was activated in breast cancer cell lines but was not activated to a significant level in breast cancer. Furthermore, the IκB homolog Bcl-3, which has been shown to function with p50 and p52 subunits to stimulate transcription (Bours *et al.*, 1993; Fujita *et al.*, 1993) and which is associated with leukemia (McKeithan *et al.*, 1997; Michaux *et al.*, 1997), was also found to exhibit increased nuclear levels in human breast cancer as compared to non-tumor tissue but was not increased in human breast cancer cell lines. Consistent with these observations, transcripts of known NF-κB-regulated genes were found to be significantly elevated in human breast cancer tissue. These data indicate that NF-κB is functionally active in human breast cancer and that a subset of NF-κB complexes, which do not include the p50/p65 heterodimer, is activated in human breast cancer. Furthermore, the results suggest that non-typical mechanisms are involved in activation of functional NF-κB in breast cancer.

Results

NF-κB binding activity is increased in breast cancer cell lines

In many instances the utilization of immortalized or transformed cell lines is widely recognized as an appropriate model to test the function of a particular transcription factor. In order to determine whether NF-κB plays a role in breast cancer, we examined several breast cancer cell lines for the activation of NF-κB. Activation of NF-κB is characterized by an increase in nuclear NF-κB, an event that can be monitored through electrophoretic mobility shift assays (EMSAs). We analysed five well characterized breast cancer cell lines: MCF7, T47D, MDA231, SKBR3 and BT474. Nuclear extracts from each of these cell lines were incubated with a radiolabeled oligonucleotide probe containing an NF-κB DNA binding site. Nuclear extracts from SKBR3 cells contain the strongest NF-κB binding complex followed by BT474, MDA231 and MCF7 (Figure 1a). Nuclear extracts from T47D cells contain very little NF-κB DNA binding activity. Similar data were obtained using different nuclear extract preparations (data not shown). To identify which NF-κB components contribute to this binding activity, we performed supershift analysis with NF-κB specific antibodies. An antibody

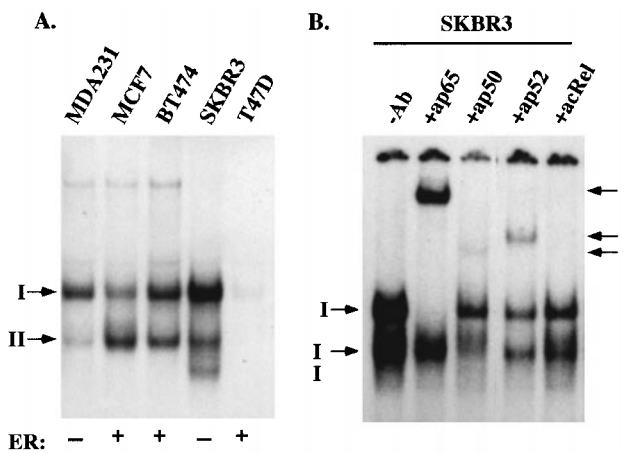

Figure 1 Analysis of NF-κB in human breast cancer cell lines. (**a**) EMSAs were performed with nuclear extracts prepared from MDA231, MCF7, BT474, SKBR3 and T47D breast cancer cell lines. Nuclear extracts (5 μg) were incubated with a ^{32}P-labeled oligonucleotide probe containing an NF-κB binding site (see Materials and methods section), and complexes were resolved on a 5% nondenaturing polyacrylamide gel. NF-κB complexes I and II are indicated with arrows. The estrogen receptor (ER) status of each cell line is indicated below each line. (**b**) Supershift analysis was performed by preincubating nuclear extract prepared from the SKBR3 cell line with various NF-κB/Rel specific antibodies for 15 min prior to the addition of the oligonucleotide probe. Arrows placed on the right side of the panel indicate supershifted complexes

specific for the p65 subunit of NF-κB, which can recognize p65 homodimers and p50/p65 heterodimers of NF-κB, supershifted complex I and was unable to shift complex II (Figure 1b). However, both complexes I and II are supershifted when a p50 antibody, which recognizes both p50 homodimers and p50/p65 heterodimers, and when a p52 antibody were used. When an antibody specific for c-Rel is added to the binding reaction there appears to be a decrease in DNA binding but no visible supershift complex is formed. The relative lack of c-Rel binding is not due to the oligonucleotide probe favoring binding of p65 over that of c-Rel, since nuclear extracts prepared form WEHI-231 cells, known to contain high levels of c-Rel binding activity, show that the probe is readily bound by c-Rel (data not shown). These data suggest that there is an increase in NF-κB DNA binding complexes in nuclear extracts of breast cancer cell lines. The greatest NF-κB binding activity occurs with complexes containing the p65 subunit of NF-κB, a classic NF-κB component.

A recent study demonstrated that estrogen receptor (ER) negative breast cancer cell lines contain an increase in constitutive NF-κB DNA binding when compared to ER-positive cell lines (Nakshatri *et al*., 1997). In our study we find that the ER negative SKBR3 cell line does show the greatest NF-κB binding complexes (Figure 1), however NF-κB binding complexes formed in the ER-positive cell line BT474 are only slightly lower. In addition, by Western analysis, the ER positive cell line MCF-7 appears to have the highest expression of both p65 and c-Rel proteins (data not shown). We have also observed by Western analysis that the ER-positive T47D cell line, which has very little NF-κB binding activity, appears to have p65 and p105 of slightly altered mobility (data not shown and see Figure 4b). The altered protein expression in these cells may contribute to the lack of NF-κB DNA binding observed in EMSA (Figure 1b), and probably has nothing to do with the ER status in that cell line. The fact that we do not observe a strong correlation with NF-κB and ER activity in these cell lines may be due to the fact that cell lines carried in culture can show great differences in the amount of NF-κB present over time. In addition, slight changes in culture media and the source of the original cell may also contribute to these differences.

Primary tumor cells from patients with breast cancer exhibit increased NF-κB binding activity

Although we have analysed NF-κB DNA binding in breast cancer cell lines, we were interested in determining the status of NF-κB activity in cells from primary breast tumors. Seventeen cases of carcinomas of the breast were received from Lineberger Comprehensive Cancer Tissue Procurement files. Sixteen were primary breast carcinomas, and one was a local recurrence. Twelve cases were pure ductal, ranging in size from 0.4–9 cm in diameter and ranging from grade 1–3. Of these cases for which steroid receptor data are available, 3/8 were ER positive and 3/6 were PR positive. Two cases were mixed ductal and lobular, ranging in size from 0.4–5 cm and ranging from grade 1–2. Of the cases of this group for which steroid receptor data are available, 2/2 were ER positive and 2/2 were PR positive. Two cases were pure lobular, ranging in size from 1.7 cm to diffuse (>20 cm) and ranging from nuclear grade 1–2. Of these cases for which steroid receptor data are available, 1/2 were ER positive, and 1/2 were PR positive.

Nuclear extracts from snap frozen tissue sections of primary breast tumors and normal adjacent breast tissue were obtained from nuclei isolated by sucrose pad purification. EMSAs were performed on nuclear extracts and DNA binding activity between tumor (T) and adjacent tissue (A) was analysed. The results show that all tumor samples contain NF-κB specific DNA binding activity (Figure 2a). Although patient 1 appears to have the highest level of NF-κB binding activity, all seven patients show a dramatic increase in NF-κB DNA binding complexes when compared to their nontumorigenic adjacent tissue. Although the absolute levels of NF-κB binding activity vary between tumorigenic patient samples, all have correspondingly higher binding activity than their adjacent tissue (Figure 2a). The level of NF-κB binding does not appear to be related to the estrogen receptor status since both ER+ and ER− samples exhibit high levels of NF-κB binding (Figure 2a).

To identify which components of NF-κB contribute to this binding activity, supershift analysis was performed with nuclear extracts from patient 7 (Figure 2b). Supershift analysis identified p50, p52 and c-Rel as the predominant NF-κB subunits contained in these binding complexes (Figure 2b). It is noted that these antibodies do not strongly reduce the major DNA/protein complex, which may be due to an opposing stabilization of the complex by the immune serum or to the inability of the complex to be efficiently recognized by the antiserum. It also remains possible that an additional, uncharacterized subunit is part of the major DNA binding activity. Antibodies to RelB did not affect the DNA/protein complex (data not shown). Surprisingly when a p65 specific antibody which recognized both p65 homodimers and p50/p65 heterodimers was added to the binding reaction only the very faint upper complex seen is supershifted. This complex appears in only three of the 17 patient samples analysed and thus does not appear to be an important factor in the overall upregulation of NF-κB activity in primary breast tumor cells. The DNA binding activity obtained with tumor nuclear extracts is specific as shown by competition with the cold probe (UV21) and with a shorter, commercially available consensus NF-κB oligonucleotide (Figure 2c). The EMSA complexes detected with breast cancer nuclear extracts do not appear to contain NF-κB binding complexes identified as complex I in breast cancer cell lines, since the tumor-derived DNA-protein complexes are faster migrating than the predominant p50/p65 heterodimer complex found in the cell lines (see Figure 2c). From these results we conclude that the increase in NF-κB DNA binding present in the nuclear extracts from breast tumor cells appears to represent an increase in p50, p52 and c-Rel but not p65. Similar results were obtained in all tumor and adjacent samples tested (data not shown). In order to confirm the statistical significance of our previous result, EMSAs were performed on additional patient tumor samples. The results from these patients demonstrate that NF-κB DNA activity is elevated in 10/10 breast tumors

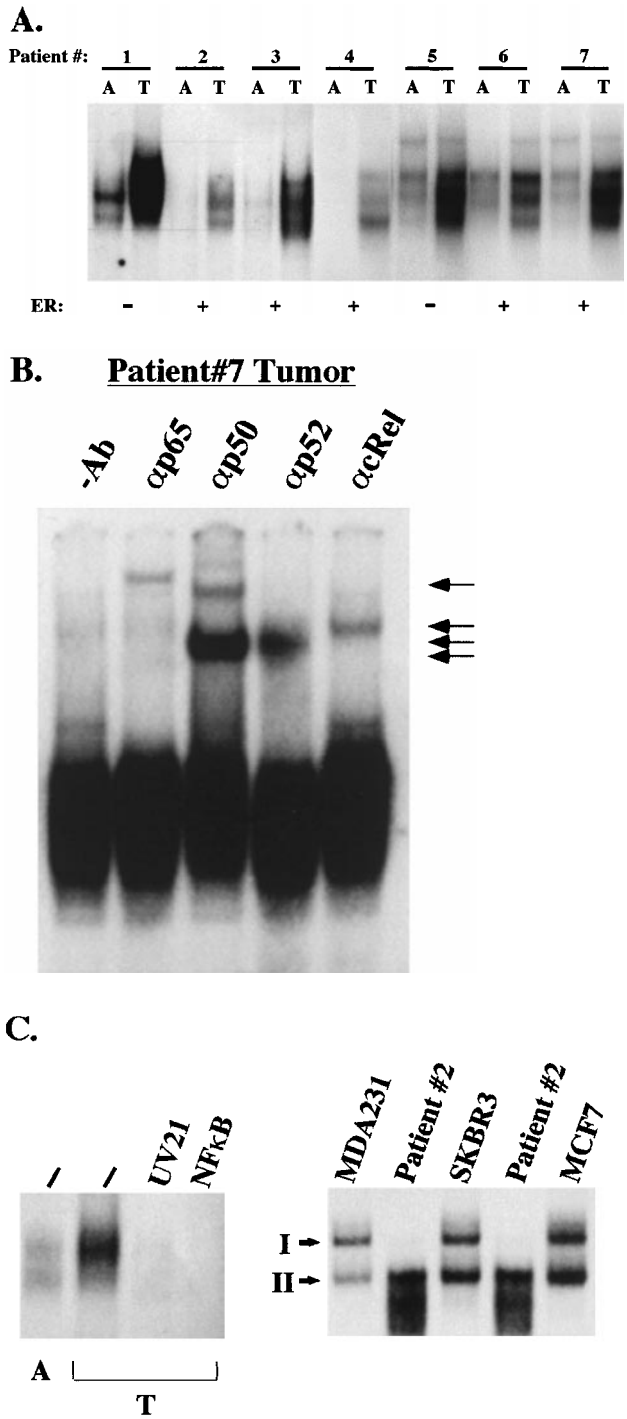

Figure 2 NF-κB DNA binding activity is elevated in breast tumors. (**a**) EMSAs were performed on nuclear extracts prepared from sucrose pad-purified nuclei from frozen tissue obtained in seven patients with breast cancer. Equivalent amounts of protein (2 μg) were used to identify DNA binding activity in tumor (T) when compared to normal adjacent tissue (**a**). Nuclear extracts were incubated with a ^{32}P-labeled oligonucleotide probe containing an NF-κB binding site. (**b**) Supershift analysis was performed on nuclear extracts isolated from tumor samples of patient 7. Extracts were preincubated for 15 min with NF-κB/Rel specific antibodies prior to the addition of probe. Upper arrows indicate supershifted complexes. (**c**) Competition analysis (left panel) was performed with cold, excess UV21 probe or with a commercially available NF-κB oligonucleotide as indicated at the top). EMSAs (right panel) were performed to compare mobilities of NF-κB complexes from breast cancer cell lines (labeled at the top) or from the breast tumor from patient 2. Complexes I and II are indicated

examined (data not shown). Thus, we have examined tumors from 17 patients and every patient sample exhibited an increase in NF-κB binding activity. Surprisingly, these NF-κB complexes do not involve significant levels of p65, the most commonly analysed NF-κB component. Although a few patients (3/17) showed an increase in p65 binding activity in breast tumors, the level of binding is significantly less than exhibited by the major complex. These data indicate that NF-κB family members other than p65 are upregulated in breast tumors.

Immunohistochemistry indicates an increase in p50/NF-κB1, p52/NF-κB2, and c-Rel proteins in breast tumors

To initially determine if p50, p52 and c-Rel are present at higher levels in breast tumor samples as compared to their normal adjacent tissues, immunohistochemistry was performed using NF-κB specific on paraffin embedded sections of normal and tumorigenic breast tissue from patients 1 and 2. The results shown in Figure 3 are from patient 1 and demonstrate an increase in p52 and c-Rel protein levels in breast tumors when compared to nontumorigenic adjacent tissue. The increased staining for c-Rel appears to be in the infiltrating ductal regions as well as in the surrounding stromal cells indicating an overall increase in staining in the tumor section. However, the staining for p52 is specifically increased in the cancerous ductal regions of the tumor section, with very little staining observed in the stromal cells, suggesting that the increase in p52 protein is seen primarily in the cancer cells. There is also an increase in staining in the tumor samples when compared to adjacent tissue using the p50 specific antibody. Staining with an antibody for the p65 subunit of NF-κB indicates that there is some difference in p65 protein levels between adjacent and tumor tissue. To determine whether the staining seen with the p65 antibody is nuclear, an antibody specific for the activated form of p65 (p65-NLS, which recognizes p65 released from IκB) was utilized and no antibody specific staining in either the adjacent or tumor sections is detectable (data not shown). Therefore, breast cancer tissues contain increased NF-κB involving the NF-κB subunits p50, p52, and c-Rel. The upregulation of p52 appeared to be more specific for tumor cells. Because of the large quantity of cytoplasmic staining for each of the samples and the high background with some of the antibodies, it is difficult to determine whether there is a corresponding increase in nuclear staining, thus we have directly measured nuclear levels of NF-κB subunits. Additionally, the immunohistochemistry does not distinguish between expression of the precursor forms (p100 and p105) and the processed forms (p50 and p52) of these NF-κB sunumits.

NF-κB subunits p50, p52 and c-Rel as well as Bcl-3 are increased in nuclei of breast cancer cells

To correlate the increases in p52, p50 and c-Rel seen in EMSA and in the immunohistochemistry experiments with actual protein levels, Western blot analysis was performed using the nuclear extracts examined in Figure 2. Nuclear proteins were electrophoresed on an SDS

polyacrylamide gel, transferered to membrane and probed with a panel of NF-κB specific antibodies. The results are shown in Figure 4a. An increase in nuclear p50, p52, and c-Rel protein levels is observed in each tumor sample when compared to adjacent nontumorigenic tissue. In agreement with our EMSA data, there was little detectable p65 protein present in either the adjacent or tumor nuclear samples from these patients. The p65 protein was not degraded in these extracts as cytoplasmic p65 was readily detectable by immunoblotting (data not shown). An antibody specific for human α-actin was used to control for equal loading. This data again differs from data obtained with breast tumor cell lines which show a significant increase in p65 protein in Western analysis (see below).

Both p50 and p52 proteins appear to be normally transcriptionally inactive, except when found in a complex with the IκB homolog Bcl-3 (Bours *et al*., 1993; Fujita *et al*., 1993). Therefore, we probed the breast cancer nuclear extracts for Bcl-3. As shown in Figure 4a, Bcl-3 was found to be significantly elevated in the nuclear extracts of each of the tumor samples analysed as compared to the normal adjacent tissue. These results provide a potential mechanism to explain transcriptional activity associated with nuclear levels of p50 and p52 (see below and see Discussion). In

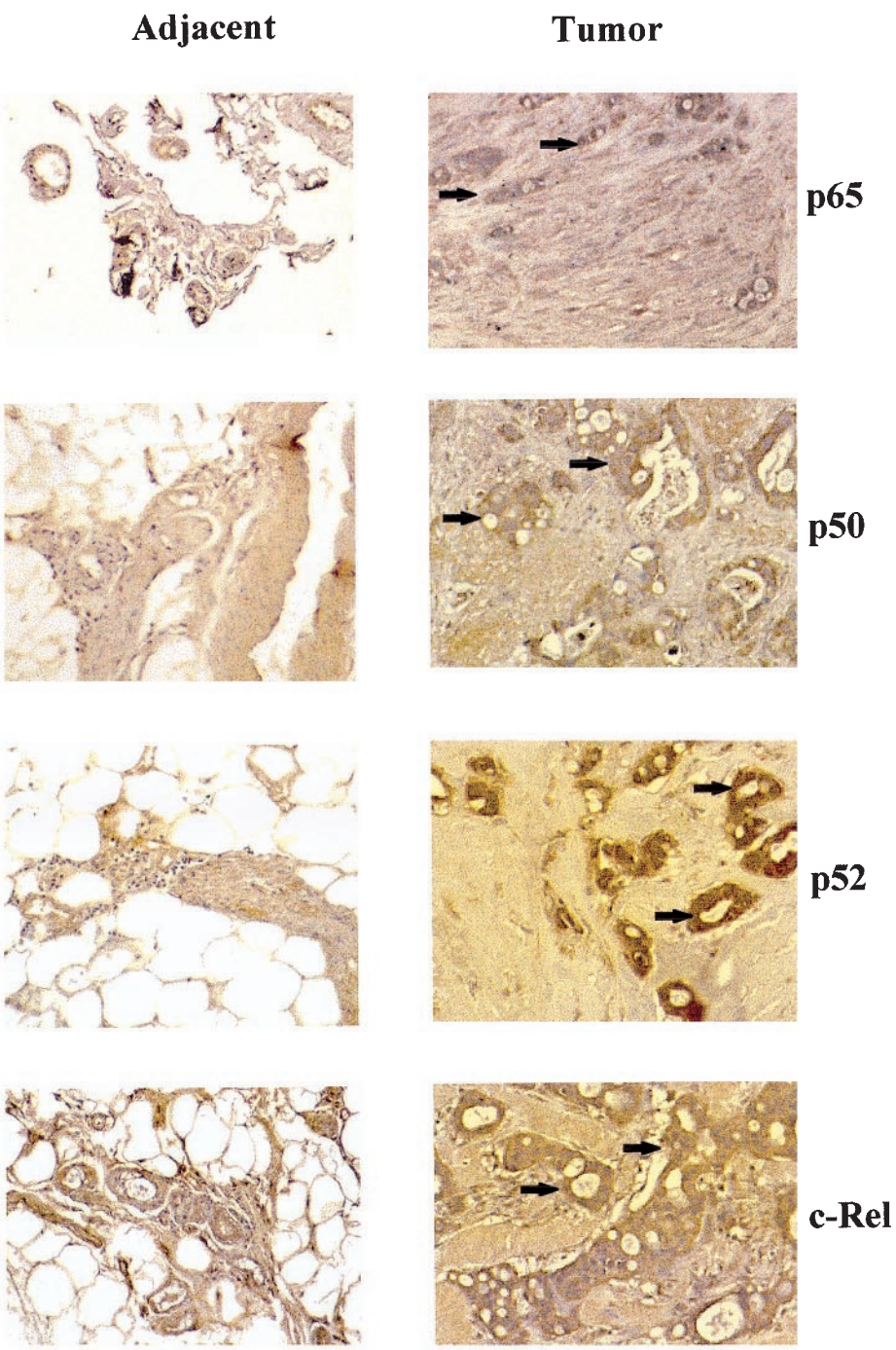

Figure 3 Immunohistochemistry analysis of breast tumor tissue for NF-κB. Sections of paraffin-embedded tumor (T) and normal adjacent tissue (A) from patient 1 were probed with p65, p50, p52 or c-Rel specific antibodies, and bound antibodies were visualized using immunoperoxidase detection. Following antibody specific staining, sections were counterstained with hematoxylin

contrast with the breast tumors, nuclear extracts derived from breast cancer cell lines exhibit elevated nuclear levels of p65 and p50, but variable expression of p52 and c-Rel (Figure 4b). As described above, there is a slower mobility of the p65 found in T47D (Figure 4b) that is apparent on longer gel migration. Additionally, breast cancer cell lines exhibited virtually no nuclear Bcl-3 (Figure 4b) which was due to low levels of Bcl-3 mRNA expression (data not shown).

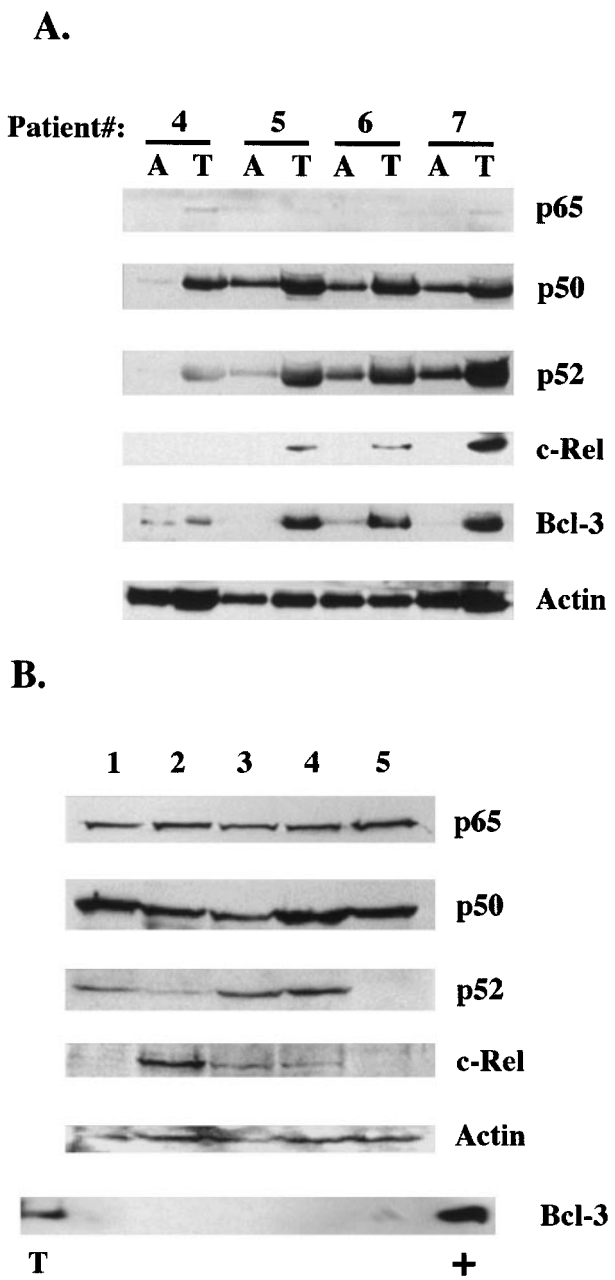

Figure 4 Levels of nuclear NF-κB proteins are elevated in breast tumors. (**a**) Nuclear extracts (24 μg) of tumors or of adjacent tissue from patients 4–7 (see Figure 1) were analysed by Western blot analysis. Proteins were fractionated on SDS polyacrylamide gels, transferred to nitrocellulose and incubated with antibodies specific for p65, p50, p52, c-Rel and Bcl-3. Normal adjacent tissue (A) and tumor tissue (T) are indicated for each patient. Blots were normalized to the level of α-actin protein. (**b**) Nuclear extracts of breast cancer cell lines were analysed by Western blot analysis for expression of the proteins shown part A. The Bcl-3 blot contained a tumor extract (T) and extracts from a 3T3 cell line expressing Bcl-3 (+) as positive controls

Activation of NF-κB regulated genes in tumorigenic tissue samples

Many of the NF-κB and IκB genes are known to be regulated by NF-κB, thus allowing for the assay of potentially increased κB-dependent gene expression in breast cancer. Thus, the p50 and p52 genes, but not the gene encoding p65, are known to be regulated by NF-κB (see Baldwin, 1996; Liptay *et al*., 1994). In order to determine whether the increase in p50 and p52 levels observed in breast cancer tumor tissue was due to an increase in mRNA, additional tumor samples were obtained and RNA analysis was performed. Enzymatic amplification by PCR is well suited for RNA analyses in this study because the limited sample amount that we were able to obtain was insufficient for more standard methods of analysis. Using the reverse transcriptase-polymerase chain reaction (RT–PCR) under limiting conditions, we generated amplified products which were electrophoresed on agarose gels. Amplified DNA levels were quantified by Southern analysis. As shown in Figure 5, an increase in mRNA from the p50 and p52 genes is observed in tumor tissue when compared to mRNA levels for these genes in adjacent tissue. Additionally, a dramatic increase in mRNA from the IκBα gene, which is known to be regulated by NF-κB (Baldwin, 1996; Ghosh *et al*., 1998), was observed in tumor samples. Presumably, the

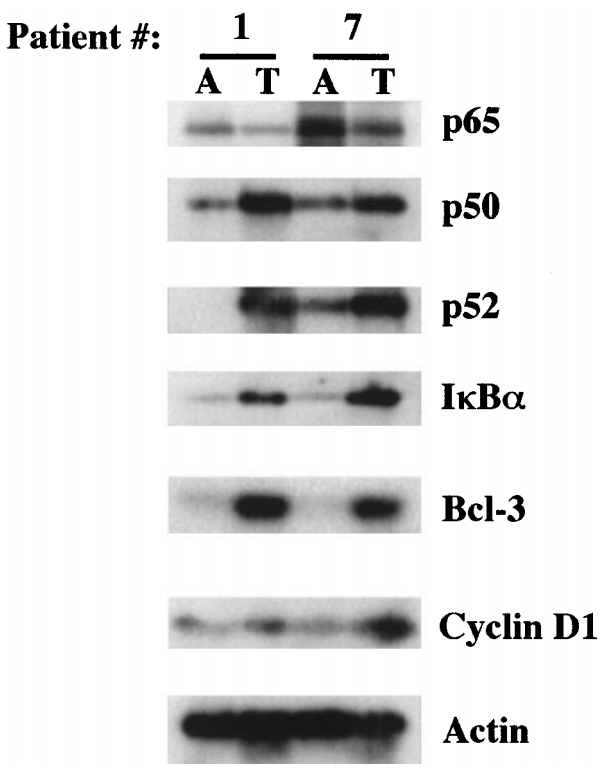

Figure 5 Increased mRNA levels of p52, p50, IκB, Bcl-3 and cyclin D1 but not p65 are seen in breast tumors. Total RNA (4 μg) was isolated from tumor and normal adjacent tissue of patients 1 and 7. RT–PCRs were performed to generate partial cDNA transcripts encoding the NF-κB family members p65, p50 and p52 as well for Bcl-3, cyclin D1, IκBα and α-actin. RT–PCR products were resolved on agarose gels and detected via Southern blotting using independently derived cDNA probes. Products were amplified 25 cycles with the exception of p65 which required an additional ten cycles for visualization. Exposure times for Southern blots ranged from 24–48 h. In all cases, RT–PCR products were of the size predicted by their nucleotide sequences

NF-κB forms activated in breast cancer can activate expression of the IκBα gene. Interestingly, upregulation of IκBα presumably would not affect the nuclear accumulation of p50 and p52 complexes. When oligos specific for p65 were used, an additional ten PCR cycles were necessary in order to visualize any product by Southern analysis. Consistent with our previous findings, there was no increase in p65 mRNA between adjacent and tumor tissue. Additionally, mRNA levels for Bcl-3, which contains NF-κB binding sites in its promoter region (see Ohno *et al*., 1990), and for cyclin D1 are elevated in breast tumor tissue as compared to adjacent tissue. Cyclin D1 was recently shown to be transcriptionally regulated by NF-κB (Hinz *et al*., 1999; Guttridge *et al*., 1999). The normalization using human α-actin shows equal PCR products for each sample. This data suggests that the increase in protein seen in Western analysis for p50, p52 and Bcl-3 is caused by an increase in mRNA level due to either the stabilization of the mRNA or, more likely, to an increase in transcription of the NF-κB regulated genes likely through the activation of functional NF-κB activity. The potential regulation of cyclin D1 gene expression in human breast cancer by NF-κB/Bcl-3 complexes is important since cyclic D1 protein expression is unregulated in the great majority of breast tumors. Preliminary data indicate that cyclin D1 can be transcriptionally activated by p50 plus Bcl-3 (S Westerheide and AS Baldwin Jr, in preparation).

Discussion

The results we obtained through EMSA and Western analysis of nuclear extracts isolated from patients with breast cancer along with immunohistochemical studies revealed a distinct pattern of activation of NF-κB subunits as compared to normal, adjacent tissue. Additionally, a different pattern of NF-κB expression was found from that seen in commonly used breast cancer cell lines and from that seen following treatment of cells with cytokines or other NF-κB inducers. Thus, breast cancer cell lines exhibit a constitutive level of NF-κB activation that consists primarily of the p50/p65 heterodimer and of the p50 homodimer. Consistent with these findings, we have made the observation that long-term culture of a variety of cell lines leads to increased levels of nuclear NF-κB consisting of the p65 subunit (data not shown). However, our data indicate that NF-κB binding complexes containing p50, p52 and c-Rel are activated (i.e., nuclear) in human breast cancers without a corresponding consistent activation of the p65 subunit. Similar to our studies, Sovak *et al*. (1997) found that two of five breast tumor samples exhibited significant p65 nuclear expression. With respect to NF-κB and its potential biological role in breast cancer, our results demonstrate that established breast cancer cell lines may not serve as a completely reliable model for this disease.

Increases in p50, p52 and c-Rel levels and binding activities are detected in breast tumors as compared to non-tumorigenic adjacent tissue. Interestingly, immunohistochemical approaches show a dramatic quantitative increase of p52 in the ductal regions of the breast cancer and not in the invading stromal fibroblasts (Figure 3). Previously, it was shown that cellular levels of the 100 kD precursor of p52 were elevated in a majority of breast cancer samples (Dejardin *et al*., 1995). However, corresponding nuclear levels of p52 were not analysed in these primary samples. Although c-Rel is known to harbor transcriptional activation domains, both the p50 and p52 subunits have not been shown to be transcriptionally active unless complexed with the IκB homolog Bcl-3 (Bours *et al*., 1993; Fujita *et al*., 1993; Schmid *et al*., 1994). Consistent with this concept, the phenotype of the Bcl-3 knockout overlaps significantly with that of the p50 and the p52 knockouts (Caamano *et al*., 1998; Franzozo *et al*., 1997, 1998; Schwarz *et al*., 1997). Importantly, we find that Bcl-3 nuclear levels are increased in breast cancer tissue as compared to the adjacent levels, which is interesting since it was published recently that Bcl-3 can function to increase nuclear levels of p50 (Zhang *et al*., 1994; Watanabe *et al*., 1997). Thus, an increase in nuclear Bcl-3 may function with increased levels of p50 and p52 to account for the enhancement in NF-κB dependent gene expression observed in human breast cancer (Figure 5). Since p50 and p52 are not thought to be directly regulated through complexes with IκBα or IκBβ, our data suggest that a pathway is active in breast cancer that functions to increase levels of certain forms of NF-κB that is independent of the normal inductive pathways that involves degradation of one of the IκB subunits. However, we cannot rule out that the increase in nuclear levels of c-Rel found in breast cancer is controlled through a pathway that involves release from a form of IκB. Our data also indicate that levels of mRNAs for p50 and p52 are increased in breast cancer, likely through the transcriptional activation of these genes by active NF-κB complexes (Baldwin, 1996; Ghosh *et al*., 1998; Liptay *et al*., 1994). This is likely to contribute to the increase in these NF-κB subunits in breast cancer. Additionally, increased mRNA levels for Bcl-3 were observed in breast tumors. However, Bcl-3 levels were barely detectable in nuclear or cytoplasmic extracts of breast cancer cell lines and Bcl-3 mRNA levels were relatively low.

Of the NF-κB subunits found activated in human breast cancer, c-Rel and p52 each have been implicated with oncogenesis (Baldwin, 1996; Gilmore, 1997; Chang *et al*., 1995). The p100/p52 gene is found translocated and rearranged in certain lymphomas such that it is overexpressed without the normal ankyrin repeats. Removal of the C-terminal ankyrin repeats of p100/p52 leads to gastric hyperplasia and to enhanced proliferative responses of lymphocytes (Ishikawa *et al*., 1997). c-Rel can be transforming when mutated and expressed in retroviral form (Gilmore, 1997) and IκBα expression can delay tumor formation in v-Rel expressing mice (Carrasco *et al*., 1997). Importantly, we find that Bcl-3 nuclear levels are increased in human breast cancer and Bcl-3 has also been found translocated [t(14;19)] and overexpressed in certain B-cell lymphomas (McKeithan *et al*., 1997; Michaux *et al*., 1997). Consistent with a role for Bcl-3 in human leukemia, it was found that Bcl-3 transgenic mice exhibit lymphadenopthy and splenomegaly (Ong *et al*., 1998).

What is the possible role for elevated NF-κB functional activity in human breast cancer? Our recent data indicated that NF-κB activation suppresses a transformation-associated apoptosis (Mayo *et al*., 1997). Thus, NF-κB may serve to suppress a similar apoptosis in developing breast cancer. Consistent with

this, Sonenshein and colleagues (Sovak *et al*., 1997) found that inhibition of NF-κB in a breast cancer cell line led to enhanced cell death. Future experiments will determine if the forms of NF-κB that are activated in human breast tumors provide a cell survival function in the tumor tissue and can activate genes known to suppress apoptosis. This process may also contribute to chemoresistance of breast tumors. Additionally, our recent data and that of others indicates that NF-κB can promote cell cycle progression through the activation of cyclin D1 promoter activity (Hinz *et al*., 1999; Guttridge *et al*., 1999) and, thus the activation of NF-κB functional activity may promote the enhanced cell proliferation and overexpression of certain cyclins that are characteristic of breast cancer (Sherr, 1996).

Materials and methods

Cell culture

All cell lines were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Core Facility at the University of North Carolina, Chapel Hill. MCF-7 cells were maintained in DMEM, T47D cells were grown in RPMI 1640 medium containing 1 × ITS (insulin-transferrin-sodium selenite; Boehringer Mannheim), BT474 cells were cultured in EMEM with ITS, MDA231 cells were grown in Iscoves medium, and SKBR-3 cells were grown in Macoys5A medium. Growth media contained antibiotics and 10% fetal calf serum obtained from Life Technologies.

Tissue samples

Lumpectomy and mastectomy samples from breast cancer patients were separated into tumor tissue and normal adjacent tissue by a pathologist. Samples were either quickly snap frozen in liquid nitrogen, or directly embedded in paraffin. A total of 17 samples were obtained, of which seven were matched sets of both tumor and normal breast tissue as determined by histological analysis.

Cell extracts and Western blotting

Nuclear extracts from each cell line were prepared as previously described (Finco *et al*., 1997). Nuclear extracts from snap frozen tissue sections were prepared by resuspending crushed, frozen samples in sucrose buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 14 mM β-mercaptoethanol and 0.1% NP40) followed by dounce homogenization. Nuclei were isolated following double sucrose pelleting through 0.88 M and 1 M sucrose solutions. For Western blotting analysis, equal amounts of protein were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were blocked in 5% milk in 1 × TBST (Tris Buffered Saline, 0.5% Tween 20) and probed with either anti-p50, anti-Bcl-3, anti-α-actin (Santa Cruz Biotechnology), anti-p65 (Rockland), or anti-p52 (Rockland) each at 1 : 1000 dilution in 1 × TBST. Blots were probed with a secondary antibody conjugated to horseradish peroxidase (Promega Corp.) at 1 : 10 000 dilution in 1 × TBST. Protein bands were visualized with an enhanced chemiluminescence detection system (Amersham Life Science).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described previously (Mayo *et al*., 1997). An oligonucleotide corresponding to an NF-κB site in the *H-2K^b* gene (5′-CAGGGCTGGGGATTCCCATCTCCCACAGTTTCACTTC-3′) was radiolabeled using α-^{32}P-dCTP and the Klenow fragment of DNA polymerase I (Boehringer Mannheim). For antibody supershift analysis, nuclear extracts were preincubated 15 min at room temperature with 1 μg antiserum before the addition of the radiolabeled gel shift probe. Antibodies used in supershift analysis are identical to those utilized for Western blotting described above.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut to 4 μm, and sections were deparaffinized in xylene, rehydrated, and probed using specific NF-κB/Rel antibodies (listed above for Western blotting). Staining was performed using the Vectastain ABC immunoperoxidase staining kit in conjugation with 3′,3′-diaminobenzidine (Vector Labs). Tissues were counterstained with Mayer's hematoxylin (Sigma), overlaid with permount mounting solution (Fisher Scientific) and placed under coverslips.

RT–PCR

Snap frozen tissue sections were homogenized in 4 M guanidinium isothiocyanate using a Brinkman Polytron (Westbury). RNA was then isolated by centrifugation through 5.7 M cesium chloride. To produce cDNA, total RNA (4 μg) and random hexamers (200 ng; Life Technologies were dentured at 80°C for 10 min and incubated for 60 min at 37°C in first strand synthesis buffer containing 10 mM DTT (Life Technologies), with 625 μM dNTP (Boehringer Mannheim), and 400 units of MMLV reverse transcriptase (Life Technologies). Resulting cDNAs were diluted 1 : 5 in dH_20 and an aliquot was combined with a mixture containing 200 nM of specific oligonucleotides, 250 μm dNTP, 1.5 mM magnesium chloride, 1 × PCR buffer, and 2.5 units of Taq polymerase (Life Technologies). Cycle conditions were the following: denaturation, 94°C for 30 s; annealing, 58°C for 30 s; and extension, 72°C for 1 min. The amount of input cDNA was adjusted first to obtain equal amounts of α-Actin product. Amplified products ranged from 450–550 bp in length. Forward (1) and reverse (2) primers used for RT–PCR were the following: p65-1, 5′-CGTGGAGGTGTATGATGATGACGTA-3′; p65-2, 5′-CTAGAATCTAGCTGGAGGGAGGCCA-3′, p50-1, 5′-TGGATCCTTCTTTGACTCATACAAT-3′; P50-2, 5′-GTCTGCTGCAGAGCTGCTTGGCGGA-3′; IκBα-1, 5′-GGGACCCTCAGCAGAGAGGAGGACC-3′; IκBα-2, 5′-GCTTTCAGTTGTTGTGATGCTGAGA-3′; cyclinD1-1, 5′-ACCCGGCCTGCTTCCACC; cyclinD1-1, 5′-GCTGCCTCCTGGAGCTGG. All products were amplified for 25 cycles with the exception of p65 which required a total of 35 cycles for visualization. Amplified products were fractionated on 1.5% agarose gels in 1 × TBE (Tris Borate-EDTA), visualized by ethidium bromide, transferred to Nytran Plus (Schleicher & Schuell) and hybridized with specific NF-κB/Rel probes, radiolabeled with [α-32P]dCTP (Pharmacia BioTech). Hybridizations were performed for 1 h at 68°C in QuickHyb Hybridization Solution (Stratagene) as recommended by the manufacturer. Blots were washed once in 2 × SSC for 15 min at room temperature followed by two washes in 0.1 × SSC for 30 min at 65°C. Exposure times ranged from 1–2 days.

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