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Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-α that can be inhibited by the tumor suppressor PTEN

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The Nuclear Factor (NF)-κB family of transcription factors controls expression of genes which promote cell growth, survival, and neoplastic transformation. Recently we demonstrated aberrant constitutive activation of NF-κB in primary human and rat breast cancer specimens and in cell lines. Overexpression of the epidermal growth factor receptor (EGFR) family member Her-2/neu, seen in approximately 30% of breast cancers, is associated with poor prognosis. Previously, Her-2/neu has been shown to signal via a phosphatidylinositol 3 (PI3)-kinase to Akt/protein kinase B (PKB) pathway. Since this signaling pathway was recently shown to activate NF-κB, here we have tested the hypothesis that Her-2/neu can activate NF-κB in breast cancer. Overexpression of Her-2/neu and EGFR-4 in Ba/F3 cells led to constitutive PI3- and Akt kinase activities, and induction of classical NF-κB (p50/p65). Similarly, a tumor cell line and tumors derived from MMTV-Her-2/neu transgenic mice displayed elevated levels of classical NF-κB. Engagement of Her-2/neu receptor downregulated the level of NF-κB. NF-κB binding and activity in the cultured cells was reduced upon inhibition of the PI3- to Akt kinase signaling pathway via ectopic expression of kinase inactive mutants, incubation with wortmannin, or expression of the tumor suppressor phosphatase PTEN. Inhibitors of calpain, but not the proteasome, blocked IκB-α degradation. Inhibition of Akt did not affect IKK activity. These results indicate that Her-2/neu activates NF-κB via a PI3- to Akt kinase signaling pathway that can be inhibited via the tumor suppressor PTEN, and is mediated by calpain rather than the IκB kinase complex. *Oncogene* (2001) **20,** 1287–1299.

Keywords: breast cancer; EGF receptor; transgenic mice; NF-κB; IκB-α; calpain

Introduction

NF-κB/Rel is a family of dimeric transcription factors characterized by the presence of a Rel homology region (RHR) of about 300 amino acids in length, which controls multiple functions including dimerization and nuclear localization. Classical NF-κB is a heterodimer composed of p65 (or RelA) and p50 (or NFκB1) subunits (Grimm and Baeuerle, 1993). The RelA subunit has potent transactivation ability, while the p50 subunit has only modest transactivation potential *in vivo*, but binds very avidly to an NF-κB element (Ballard *et al*., 1992; Grimm and Baeuerle, 1993; La Rosa *et al*., 1994). Many genes are regulated by NF-κB (Grimm and Baeuerle, 1993). For example, we demonstrated that the c-*myc* oncogene is potently transactivated by NF-κB/Rel factors (Duyao *et al*., 1992; Kessler *et al*., 1992; La Rosa *et al*., 1994). In most cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to one of the specific inhibitory proteins termed IκBs of which IκB-α is the paradigm. During activation of NF-κB by such extracellular stimuli as TNF and IL-1, phosphorylation of IκB-α by the IκB kinase complex targets the inhibitor protein for degradation via the proteasome pathway. The IκB kinase complex contains two IκB kinases, IKKα (or IKK-1) and IKKβ (or IKK-2). Activation of the IκB kinase complex is mediated via phosphorylation of either IKKα or IKKβ (DiDonato *et al*., 1997; Mercurio *et al*., 1997; Regnier *et al*., 1997; Woronicz *et al*., 1997). IκB-α is then recruited in the IκB kinase complex, where it is phosphorylated by the functional IKKα/IKKβ heterodimer at serine residues at positions 32 and 36 (reviewed in Mercurio and Manning, 1999). This phosphorylation is followed by ubiquitination and rapid degradation through the proteasome pathway (Brown *et al*., 1995; Chen *et al*., 1996; DiDonato *et al*., 1996). This pathway is not universal, however, and proteasome-mediated degradation of IκBα has been ruled out recently in the case of NF-κB induction by oxidative stress and in its constitutive expression in early mature B cells (Fields *et al*., 2000; Miyamoto *et al*., 1998; Schoonbroodt *et al*.,

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2000). In these cases, degradation of IκB-α by calpain has been implicated.

Several years ago, we (Sovak *et al*., 1997) and others (Nakshatri *et al*., 1997) demonstrated that breast cancer cell lines and primary breast cancer specimens are typified by aberrant constitutive activation of NF-κB. Specifically, human breast tumor cell lines, and the majority of primary human breast tumor tissue samples and mammary tumors induced upon carcinogen treatment of female Sprague-Dawley rats were found to constitutively express high levels of nuclear NF-κB/Rel. In contrast, untransformed breast epithelial cells and normal rat mammary glands contained the expected low basal levels. The Her-2/neu (or c-erbB-2) oncogene, the second member of the epidermal growth factor (EGF) receptor family (EGFR-2), encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu, which has been seen in approximately 30% of breast cancers, is associated with poor overall survival (Hortobagyi *et al*., 1999). In particular, it has been found associated with increased metastatic potential and resistance to chemotherapeutic agents. Transgenic mice overexpressing Her-2/neu develop focal mammary tumors after a long latency, whereas, multi-focal tumors appeared after a significantly reduced time frame upon overexpression of both Her-2/neu and TGF-α, a ligand for this EGFR member (Guy *et al*., 1992; Muller *et al*., 1996). Recent work has implicated the phosphatidylinositol 3 (PI 3)-kinase and serine/threonine kinase Akt/protein kinase B (PKB) in Her-2/neu signaling (Adam *et al*., 1998; Ignatoski *et al*., 2000). PI3-kinase has been shown to play an important role in proliferation and cell survival induced by many cytokines (Miyajima *et al*., 1999). The tumor suppressor PTEN is a multifunctional phosphatase, which is capable of dephosphorylating products of PI3-kinase (Di Cristofano and Pandoli, 2000). PI3-kinase signaling is mediated via activation of the serine/threonine kinase Akt/protein kinase B (PKB) (Kennedy *et al*., 1997). PI3-kinase signaling has also been linked to the induction of NF-κB (Arsura *et al*., 2000; Beraud *et al*., 1999). For example, we showed that PI3-kinase signaled NF-κB activation via IKKα in Ras-transformed rat liver epithelial cells (Arsura *et al*., 2000). Furthermore, Akt was recently shown to activate NF-κB via the IKK complex in response to TNF or PDGF stimulation (Ozes *et al*., 1999; Romashkova and Makarov, 1999). Thus, here we have examined the role of Her-2/neu in mediating induction of NF-κB in breast cancer. We demonstrate NF-κB induction in breast cancer cell lines and primary mouse tumors. Furthermore, activation occurs via a PI3-kinase to Akt pathway that can be repressed by the tumor suppressor PTEN. Surprisingly, this activation does not appear to be mediated via the IKK complex proteasome pathway but rather our results implicate calpain in the basal degradation of IκB-α.

Results

Overexpression of Her-2/neu + EGFR-4 induces NF-κB

To determine whether overexpression of Her-2/neu can lead to induction of NF-κB, we characterized Ba/F3 clones stably expressing either EGFR-1 (Ba/F3-1), EGFR-2 (Her-2/neu) (Ba/F3-2), or combinations of EGFR-1 + Her-2/neu (Ba/F3-1 + 2), and Her-2/neu + EGFR-4 (Ba/F3-2 + 4). Ba/F3 cells transfected with the empty vector LXSN DNA were employed as control (termed parental Ba/F3 here). We first confirmed the previously observed basal tyrosine phosphorylation activity of the Her-2/neu + EGFR-4 receptor signaling using immunoblotting (Riese *et al*., 1996). Whole cell extracts (WCEs) were prepared from the four clones, as well as from parental Ba/F3 cells. Samples were subjected to an immunoblot assay with an antibody specific for phosphotyrosine Her-2/neu (Figure 1a). As expected, tyrosine phosphorylation of the Her-2/neu protein was observed in Ba/F3-2 + 4 cells overexpressing Her-2/neu + EGFR-4 proteins. In addition, low levels of activity were seen in Ba/F3-1 cells, expressing EGFR-1. Equal loading was confirmed by immunoblot analysis of the same filter for Akt protein levels. Thus, as seen previously (Riese *et al*., 1996), overexpression of the Her-2/neu + EGFR-4 receptors in Ba/F3 leads to elevated kinase activity and resultant basal phosphorylation of Her-2/neu.

To assess NF-κB/Rel binding levels, nuclear extracts were prepared from the Ba/F3 lines and used in EMSA. An oligonucleotide containing the NF-κB element upstream of the c-*myc* promoter, which binds all Rel family members (La Rosa *et al*., 1994) was used as probe. Nuclear extracts from the parental Ba/F3 cells and from the Ba/F3-2 cells displayed low levels of two NF-κB binding complexes (Figure 1b). Higher levels of band 1 were seen in the Ba/F3-1, Ba/F3-1 + 2 and Ba/F3-2 + 4 lanes. Interestingly, an increased level of band 2 was observed predominantly in the Ba/F3-2 + 4 cell extract, and to a lesser extent in the Ba/F3-1 lane (better seen with a longer exposure). Equal loading was confirmed by analysis for the ubiquitously expressed Sp1 protein (Figure 1b, bottom panel). Similar profiles were seen previously in wild-type Ba/F3 cells and bands 1 and 2 were identified as p50 homodimers, and classical NF-κB (p50/p65), respectively (Besancon *et al*., 1998; Jeay *et al*., 2000). To confirm the identity of the subunits within the Ba/F3-2 + 4 cells, supershift EMSA was performed (Figure 1c). Addition of an antibody that preferentially recognizes p50 in a homodimer complex, eliminated band 1 and reduced band 2. Addition of an antibody against p65 selectively reduced band 2. Taken together, these results indicate that band 1 contains p50 homodimers and band 2 is a heterodimer of p50/p65 or classical NF-κB. Thus, overexpression of the Her-2/neu + EGFR-4 receptors in Ba/F3 cells leads to elevated basal phosphorylation of Her-2/neu and to activation of classical NF-κB.

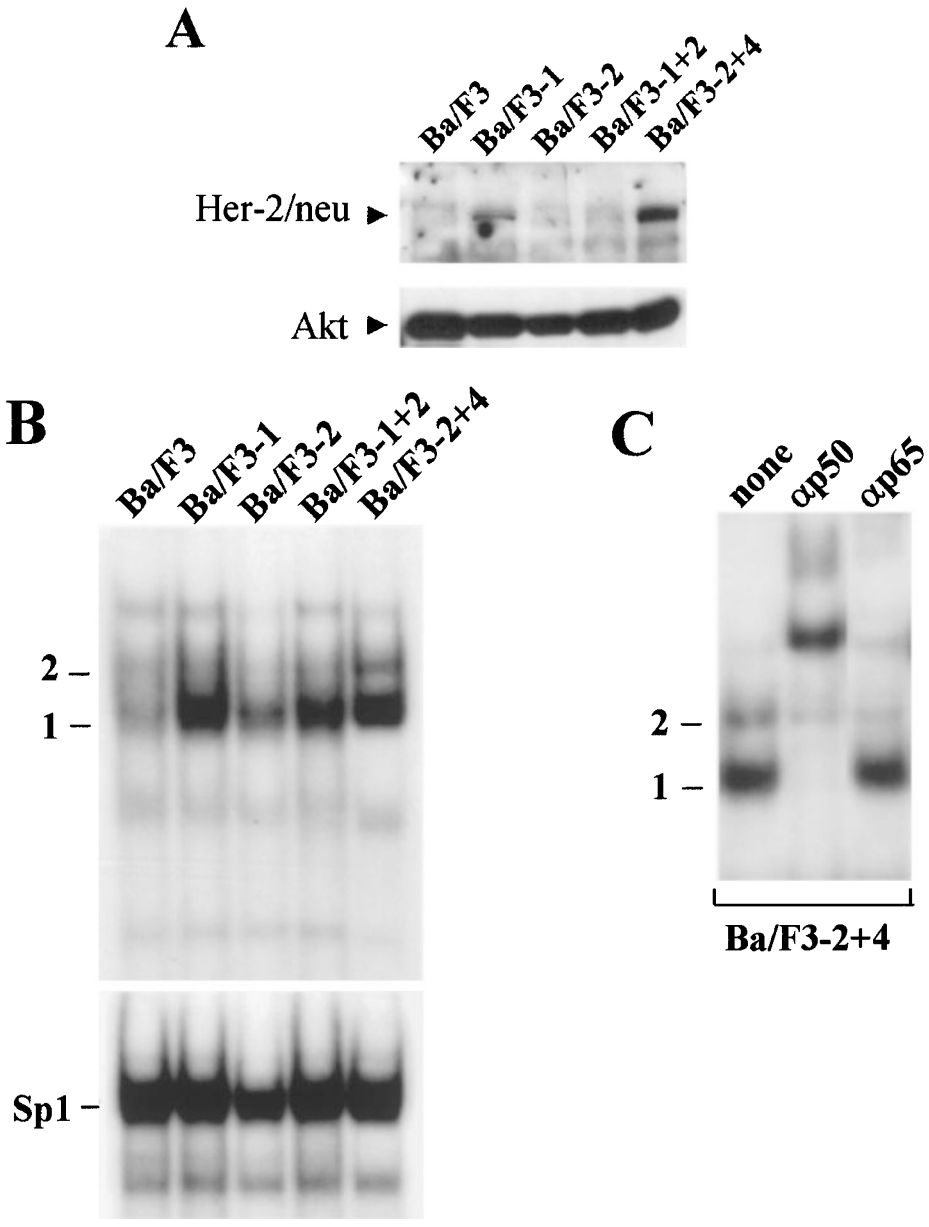

Figure 1 NF-κB activation in Ba/F3 cells expressing various EGF receptors. (**a**) Tyrosine phosphorylation of Her-2/neu is highest in Her-2/neu + erbB-4 double recombinant Ba/F3 clones. Total proteins were isolated with RIPA buffer from Ba/F3 clones, and samples (60 μg) were analysed by immunoblotting with an anti-phosphotyrosine Her-2/neu antibody (top panel), and an AKT antibody, as control for equal loading (bottom panel). Ba/F3 cell lines derived by transfection with vector LXSN DNA only, Ba/F3; vectors expressing EGFR, Ba/F3-1; Her-2/neu, Ba/F3-2; EGFR + Her-2/neu, Ba/F3-1 + 2; Her-2/neu + erbB-4, Ba/F3-2 + 4. (**b**) HER-2/neu + erbB-4 double recombinant Ba/F3 cells display elevated levels of NF-κB/Rel binding levels. Nuclear extracts were prepared from the Ba/F3 clones and used in EMSA with an oligonucleotide containing the URE NF-κB element upstream of the c-*myc* promoter, as probe (top panel) or with an Sp1 oligonucleotide, as a loading control (bottom panel). (**c**) Her-2/neu + erbB-4 double recombinant Ba/F3 cells express classical NF-κB. Supershift EMSA was performed with nuclear extracts from the Her-2/neu + erbB-4 transfected Ba/F3-2 + 4 cells in the absence (none) or presence of antibodies specific for NF-κB subunit: p50, sc-114 (αp50), or p65, sc-372 (αp65). The two major complexes, labeled bands 1 and 2, were identified as p50 homodimers and p50/p65, respectively

Activation of NF-κB occurs via a PI3-kinase signaling pathway

Her-2/neu has been shown to signal via PI3-kinase, which has been implicated in NF-κB induction (Arsura *et al*., 2000). To test for involvement of this pathway, we employed wortmannin, a potent PI3-kinase inhibitor (Arcaro and Wymann, 1993). Ba/F3-2 + 4 cells were incubated for 24 h in the presence of 100 nM wortmannin, that had been dissolved in DMSO, or the equivalent amount of carrier solution. Wortmannin treatment resulted in a significant decrease in NF-κB binding compared to treatment with DMSO alone (Figure 2a). Equal loading of Sp1 confirmed the

selectivity of the observation. Since one of the important downstream targets of PI3-kinase is Akt, we next tested for phosphorylation of this kinase (Figure 2b). A significant decrease in Akt phosphorylation was noted upon 24 h treatment with 100 nM wortmannin. Densitometry indicated wortmannin-treated cells displayed 60% of the Akt phosphorylation seen in control cells. Thus, NF-κB activation in the Ba/F3-2+4 cells appears to be mediated via a PI3-kinase to Akt signaling pathway.

MMTV-Her-2/neu tumor cell line expresses elevated levels of classical NF-κB

The NF639 cell line was established from a mammary tumor that arose in the MMTV-Her-2/neu transgenic mouse (Elson and Leder, 1995). To test for NF-κB activation, EMSA was performed using nuclear extracts from these cells compared to non-tumorigenic MCF-10F HMECs, which express only low basal levels of classical NF-κB (Sovak *et al*., 1997) (Figure 3). The level of binding was significantly higher in the NF639 line compared to MCF-10F cells. The specificity of the complexes was confirmed using competition assays and IκB-α-GST protein, a specific inhibitor of NF-κB DNA binding. Successful competition was observed with the NF639 cell extracts upon addition of excess oligonucleotide containing wild-type but not mutant URE NF-κB elements (Figure 3). Furthermore, addition of IκB-α GST fusion protein, greatly reduced formation of bands 1 and 2. Bands 1 and 2 in the human MCF-10F line have been identified as p50 homodimer and p50/p65 classical NF-κB, respectively (Kim *et al*., 2000). Addition of an antibody against the p50 subunit greatly reduced bands 1 and 2, while addition of an antibody against p65 selectively reduced band 2. In contrast, addition of an antibody against c-Rel subunit had no detectable effect on these specific complexes, while a p52 specific antibody caused a slight reduction in band 1. Thus, antibody supershift analysis identified band 2 in the NF639 nuclear extract as consisting of heterodimers of p50 and p65, while band 1 contains p50 and possibly some p52 protein, as well.

Using transient transfection analysis of wild-type vs mutant NF-κB element driven CAT reporter constructs (E8 vs mutE8), we previously demonstrated that MCF-

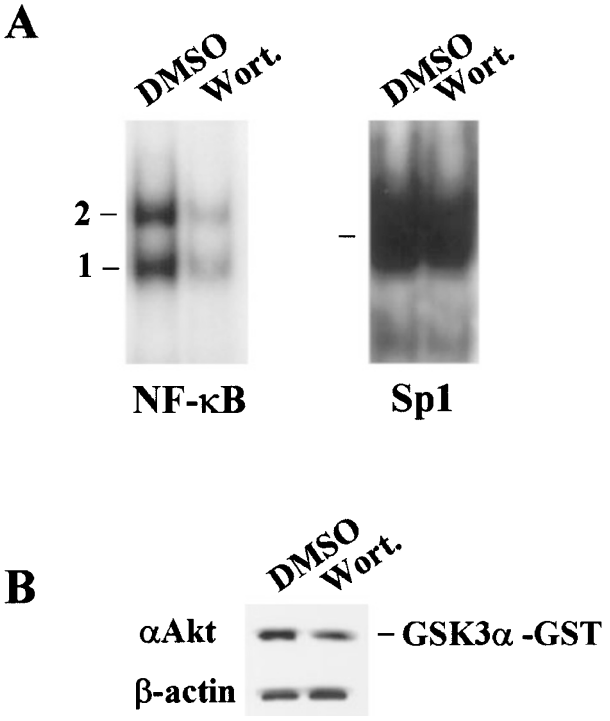

Figure 2 Wortmannin reduces NF-κB binding and phosphorylation of Akt. Her-2/neu+erbB-4 transfected Ba/F3-2+4 cells were incubated for 24 h in the presence of PI3-kinase inhibitor wortmannin (100 nM) dissolved in DMSO or the equivalent volume of DMSO alone. (**a**) Nuclear extracts were isolated and samples (5 μg) subjected to EMSA for NF-κB (left panel) and Sp1 (right panel), as loading control. (**b**) Total proteins were isolated with lysis buffer. Samples (60 μg) were immunoprecipitated overnight with a phospho-Akt antibody immobilized on agarose beads, and bound proteins used in a kinase assay with 1 μg GSK3α-GST protein as substrate. Phosphorylated GSK3α was identified by immunoblotting. Alternatively, samples (60 μg) were subjected to immunoblotting for β-actin, as a control for equal protein loading

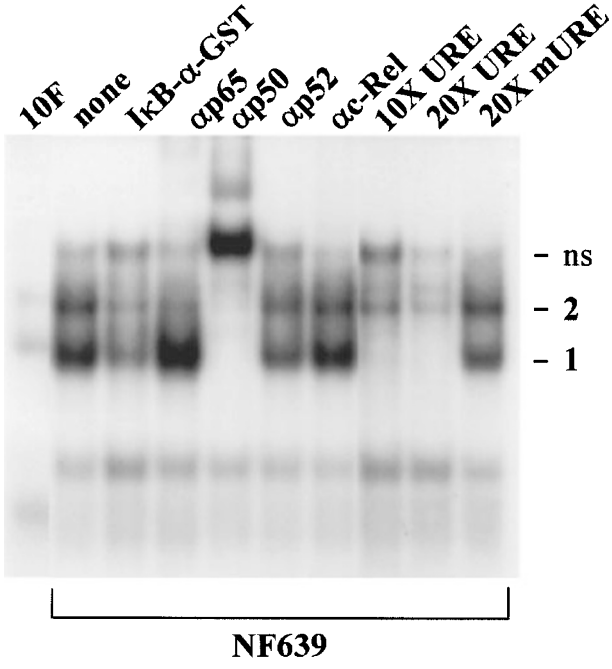

Figure 3 MMTV- Her-2/neu mammary tumor derived cell line NF639 expresses classical NF-κB. Samples of NF639 cell nuclear extracts (5 μg) were incubated overnight at 4°C in the absence (none), or the presence of 1 μg IκB-α-GST protein, or 1 ul antibody against the p65, p50, p52, or c-Rel NF-κB subunits, as indicated. Alternatively, a nuclear extract from MCF-10F (10F) cells was used. EMSA for NF-κB was performed, as described in Materials and methods. To test for specificity of binding, the binding reaction with the NF639 cell nuclear extract was incubated with 10× or 20× excess unlabeled wild-type (URE) or mutant URE oligonucleotide (mURE), as indicated. The positions of two specific complex, labeled bands 1 and 2, as well as, a non-specific complex (ns), are as indicated. (All lanes are from the same gel)

10F cells display very little NF-κB transactivation capability, consistent with the low levels of binding seen (Kim *et al*., 2000). In particular, MCF-10F cells showed a minimal induction of E8 activity over the mutE8 of approximately 1.7-fold ± 0.6. Transient transfection analysis of the NF639 cells was performed similarly using the E8 and mutE8 TK promoter-CAT reporter vectors. A ninefold ± 0.4 higher level of activity was obtained with E8 compared with mutE8 vector. Thus, the NF639 cells display elevated levels of NF-κB binding and transactivation potential.

MMTV-Her-2/neu mouse mammary tumors express elevated levels of NF-κB

We next asked whether mouse mammary tumors driven by MMTV-Her-2/neu express constitutive nuclear NF-κB. Nuclear extracts were prepared from several tumors and from histologically normal mammary glands from the same animals. As seen previously in the rat (Sovak *et al*., 1997; Kim *et al*., 2000), a normal mammary gland expresses only low levels of NF-κB (Figure 4a and data not shown). Tumors displayed elevated levels of NF-κB (Figure 4a), consistent with the EMSA of the NF639 line seen above (Figure 3). Two specific complexes were apparent, as judged by competition analysis with excess unlabeled oligonucleotide, which were labeled bands 1 and 2 (Figure 4b). Antibody supershift analysis identified bands 1 and 2 as p50 homodimers and p50/p65 heterodimers, respectively, consistent with the studies on the NF639 line (Figure 4b). EMSA with oligonucleotides containing binding sites for PU.1, and TCF-1 indicate the NF-κB binding in the tumor sample nuclear extracts was not likely due to contamination of B lymphocytes, neutrophils, mast or myeloid cells, or of T lymphocytes, respectively. Thus, mammary tumors from MMTV-Her-2/neu mice, as well as the cell lines derived from these tumors, express constitutive nuclear NF-κB.

Inhibition of the PI3-kinase to Akt signaling pathway reduces NF-κB levels

We next sought to determine the involvement of the PI3-kinase/Akt signaling pathway in the activation of NF-κB in the NF639 line. We first tested whether specific inhibition of the pathway affects NF-κB binding. NF639 cells were transiently transfected with vectors expressing either a kinase inactive version of PI3-kinase (PI3KΔ85) or of Akt (AktΔK), which function as dominant negatives. Alternatively vectors expressing either wild-type (Akt) or a membrane form of Akt, which is constitutively active (AktM) were similarly transfected to determine whether increasing the level of this kinase will enhance activation of NF-κB in the NF639 line. The transfection efficiency of the NF639 cells was approximately 60% as judged by green fluorescent protein (data not shown). Expression of either the dominant negative PI3-kinase or Akt caused a significant reduction in NF-κB binding (Figure 5a), implicating this pathway in activation of this transcription factor in the NF639 MMTV-Her-2/neu cells. Expression of Akt had only marginal effects

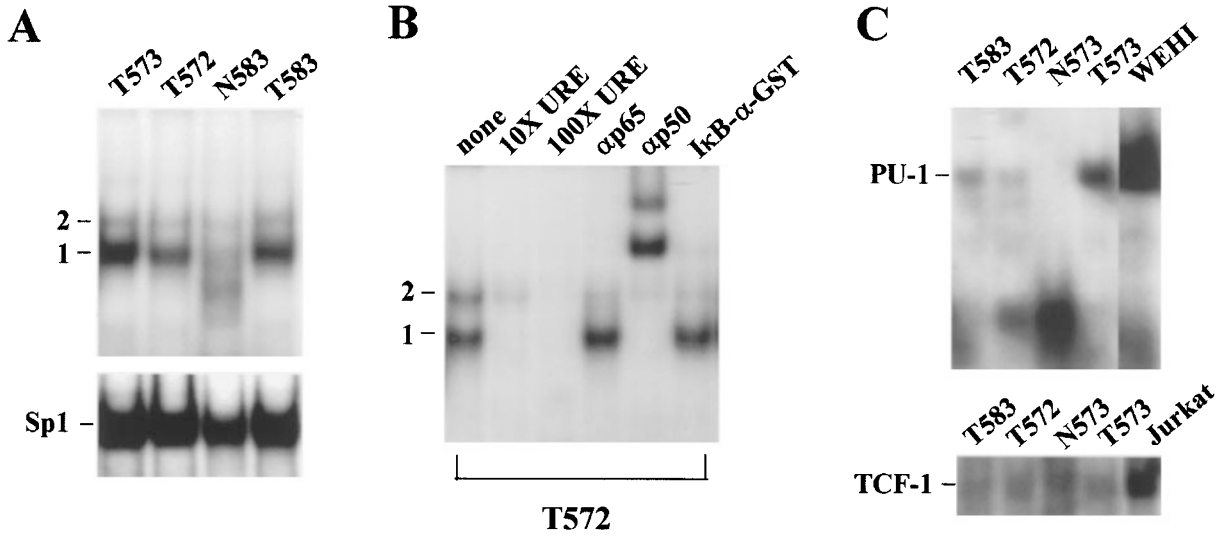

Figure 4 MMTV-Her-2/neu tumors display elevated NF-κB binding. Nuclear extracts were prepared from the indicated histologically normal mammary glands (N) and breast tumors (T) from MMTV-Her-2/neu mice. (**a**) NF-κB EMSA. Samples of nuclear extracts (5 μg) were subjected to EMSA for NF-κB and Sp1, as loading control. (**b**) Antibody supershift. Samples of nuclear extracts (5 μg) from the T572 tumor were incubated overnight at 4°C in the absence (none) or the presence of 1 ul antibody against the p65 or p50 NF-κB subunits, or 1 μg IκB-α-GST protein, as indicated. EMSA for NF-κB was performed, as described in Materials and methods. To test for specificity of binding, samples were incubated with 10× or 100× excess unlabeled wild-type (URE). The positions of two specific complexes are as indicated. (**c**) PU.1 and TCF-1 EMSA. Samples of nuclear extracts (5 μg) were subjected to EMSA for PU.1 and TCF-1. Where indicated, samples (5 μg) of nuclear extract of WEHI 231 B cells (WEHI) and Jurkat T cells (Jurkat) were added as controls for positive binding

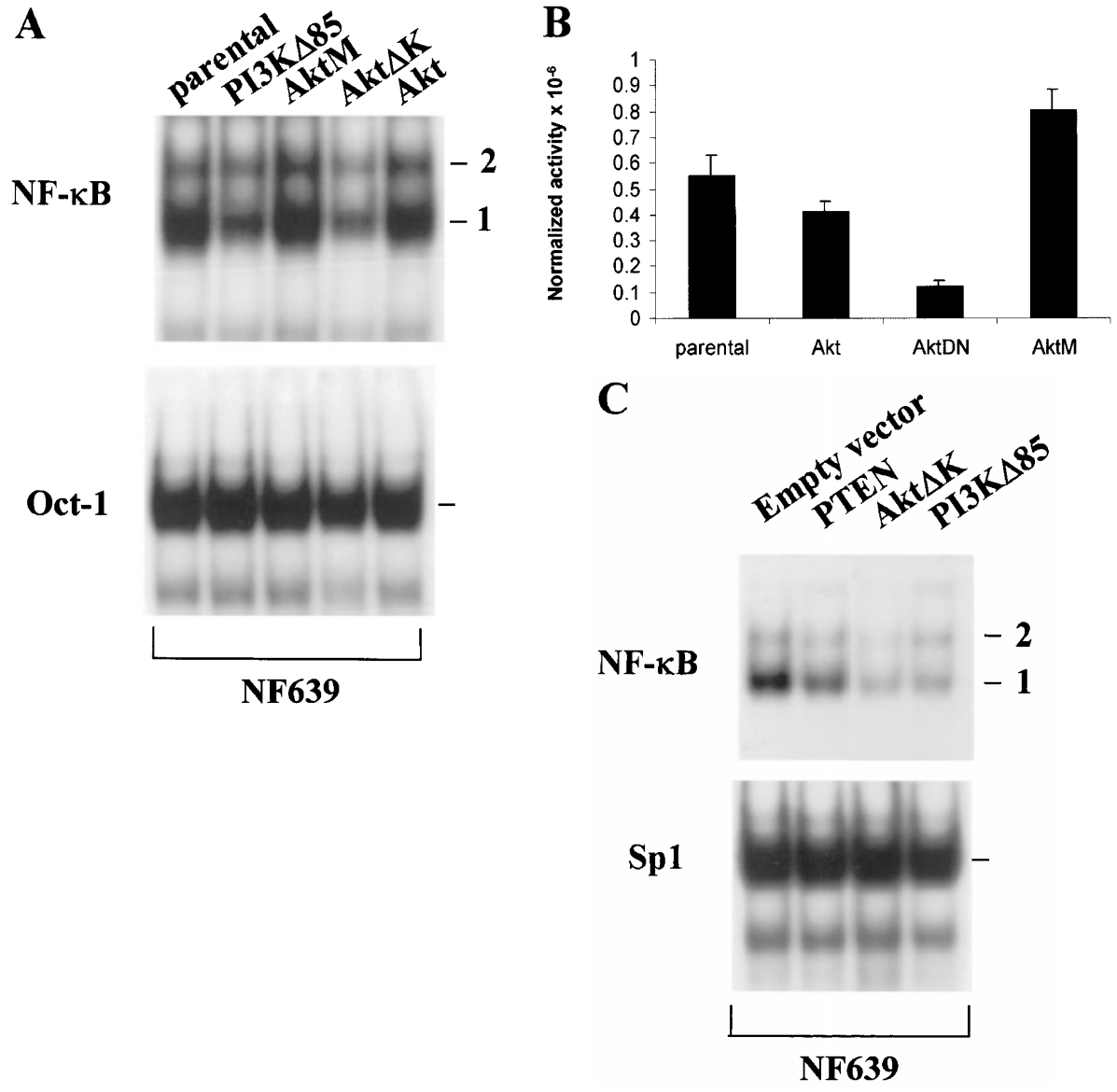

Figure 5 Inhibition of PI 3-kinase and Akt reduce NF-κB activity. (**a**) Cultures (P100) of NF639 cells were transfected with 9 μg of SRα, parental vector or of vectors: PI3KΔ85 (dead kinase), AktM (membrane kinase), AktΔK (dead kinase), Akt (wt Akt). After 48 h, nuclear extracts were prepared and samples (5 μg) used in EMSA with oligonucleotides containing elements for NF-κB or Oct-1, as control for equal loading. Positions of the two major NF-κB bands and the one Oct-1 complex, are as indicated. (**b**) Cultures (P35) of NF639 cells were transfected, in triplicate, with 1 μg NF-κB-element driven luciferase construct in the presence of 2 μg either pCMV empty vector (parental), or of vectors: AktΔK (AktDN), Akt, or AktM, and 1 μg SV40βgal. After 36 h, extracts were prepared and samples, normalized for β-galactosidase activity, were assayed for luciferase activity. The average values are presented +/− the standard deviation. (**c**) Cultures of NF639 cells were transfected, as in part (**a**), with either pCMV empty vector, or the indicated expression constructs. After 24 h, nuclear extracts were prepared and samples (5 μg) used in EMSA with oligonucleotides containing elements for NF-κB or Sp1, as control for equal loading

on NF-κB binding, while expression of the myristylated AktM form led to increased NF-κB binding (Figure 5a). Equal loading was confirmed via EMSA for the Oct-1 protein (Figure 5a). We next assessed the effects of these vectors on NF-κB activity, using co-transfection analysis of an NF-κB element driven luciferase construct (Figure 5b). Expression of the dominant negative Akt caused an over 80% reduction in NF-κB activity while expression of the constitutively active myristylated AktM form resulted in elevated NF-κB activity, and the wild-type Akt had little effect on activity or binding (Figure 5b). Thus, the changes in NF-κB binding were paralleled by changes in activity. Taken together, these findings indicate a PI3-kinase/Akt pathway plays a role in the constitutive activation of NF-κB in the MMTV-c-neu NF639 cells.

The PTEN lipid phosphatase, which is the most highly mutated tumor-suppressor gene in breast cancer, has been shown to functionally down-modulate PI3-kinase activity (reviewed in Di Cristofano and

Pandolfi, 2000). Thus we next asked whether PTEN expression affects NF-κB binding. NF639 cells were transfected with a PTEN expression vector or with the dominant negative PI3-kinase and Akt expression vectors for comparison and EMSA performed. As seen in Figure 5c, expression of PTEN reduced the level of NF-κB binding, although, to a lesser extent than seen with the kinase inactive PI3-kinase and Akt proteins. Equal loading was confirmed by analysis for Sp1 binding. Thus, PTEN reduces the activation of NF-κB seen in the Her-2/neu transformed NF639 line.

Engagement of the Her-2/neu receptor reduces NF-κB levels

Anti-neu antibody treatment of breast cancer cells *in vitro* has been found to cause a reversion of transformed phenotype (Drebin *et al*., 1985; Pegram *et al*., 1998). Since NF-κB has been implicated in regulation of genes that control transformed phenotype of breast cancer cells, including c-*myc* (Duyao *et al*., 1992; Kessler *et al*., 1992; La Rosa *et al*., 1994), we next evaluated the effects of this treatment on NF-κB. NF639 cells were incubated with an antibody that recognizes rat Her-2/neu (αneu) for 24 h, and nuclear extracts subjected to EMSA for NF-κB and Sp1, as a control for equal loading. As seen in Figure 6a, the anti-Her-2/neu antibody caused a significant reduction in NF-κB binding. To verify the efficacy of the antibody treatment on the Akt signaling pathway, extracts were subjected to an Akt kinase assay. Whole cell extracts were immunoprecipitated with a monoclonal anti-Akt, which preferentially recognizes phosphorylated protein. GSK3α-GST was then used as substrate with the resulting immunoprecipitated Akt, and phosphorylated material identified by immunoblot analysis for phosphorylated GSK3α-GST protein (Figure 6b). Treatment with the Her-2/neu antibody resulted in a decrease in phosphorylated GSK3α-GST, demonstrating receptor engagement causes decreased Akt kinase activity. Immunoblotting of the WCE for β-actin demonstrated the specificity of the decrease in Akt kinase activity (Figure 6b). Overall, these results confirm the ability of anti-Her-2/neu treatment to reduce NF-κB levels in breast cancer cells, and suggest involvement of Akt.

IKK pathway is not involved in the NF-κB induction by Her-2/neu

To explore the role of the IκB kinase/proteasome pathway, NF639 cells were incubated in the presence of either 40 μg/ml of MG132, a specific inhibitor of the proteasome pathway or carrier DMSO, as control. After 30 min, cycloheximide was added and WCEs prepared at the times indicated and subjected to immunoblotting for IκBα expression (Figure 7a). Addition of MG132 had only a very modest affect on decay of IκB-α. To verify the effectiveness of the MG132, the blot was probed for expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ protein, which

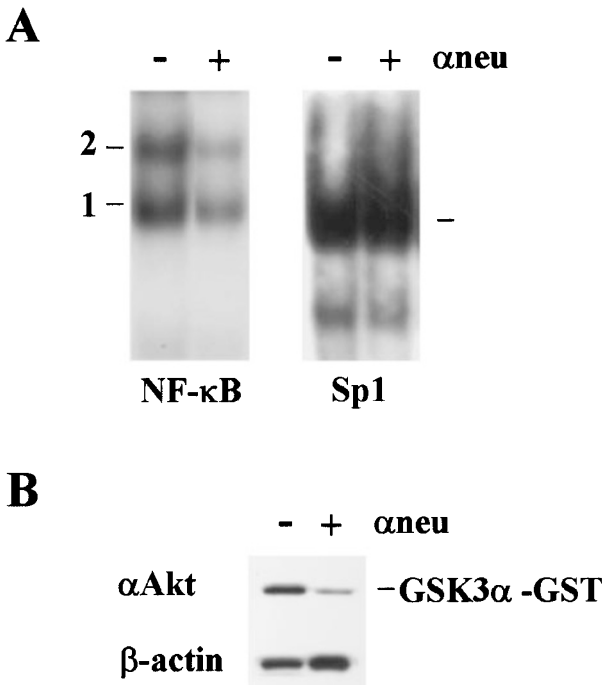

Figure 6 Engagement of the Her-2/neu receptor reduces NF-κB binding. Cultures (P100) of NF639 cells were incubated in the absence or presence of 1 μg anti-HER-2/neu (αneu) antibody for 24 h and nuclear and whole cell extracts prepared. (**a**) EMSA. Samples of nuclear extracts (5 μg) were subjected to EMSA for NF-κB and Sp1, as loading control. (**b**) Akt kinase assay. Samples of whole cell extracts (80 μg) were incubated with an Akt antibody, and the resulting immunoprecipitated Akt protein subjected to kinase assay using GSK3α-GST, as substrate (as described in the legend to Figure 2). Phosphorylated GSK3α was identified by immunoblotting. Samples (80 μg) of the whole cell extracts were analysed for β-actin, as control

is degraded via the proteasome (Tam *et al*., 1997) (Figure 7b). Turnover of p27 protein was completely ablated by the addition of MG132. Similarly, addition of MG132 blocked turnover of the c-Myc protein (data not shown), which is also mediated by the proteasome (Gregory and Hann, 2000). Thus, basal turnover of IκB-α largely does not appear to be mediated by the proteasome in NF639 cells.

To further assess the potential role of the proteasome, NF639 were transfected with a Ser32/36 to Ala double mutant version of human IκB-α (A32/36). This protein cannot be phosphorylated and subsequently degraded by the proteasome pathway. Decay of the A32/36 double mutant protein was followed using a human-specific IκB-α antibody (Figure 7c). The rate of decay was quite similar to that of the endogenous IκB-α seen above, suggesting again that decay is mediated via a non-proteasome pathway.

We next assessed the role of the IKK kinase complex in basal NF-κB expression. NF639 were transiently transfected with the vector expressing AktΔK to inhibit Akt or with the parental vector, and analysed for effects on IKKα and IKKβ activity. Alternatively cells were transfected with vectors expressing AktM or wild-type Akt. Extracts were prepared, and samples (200 μg)

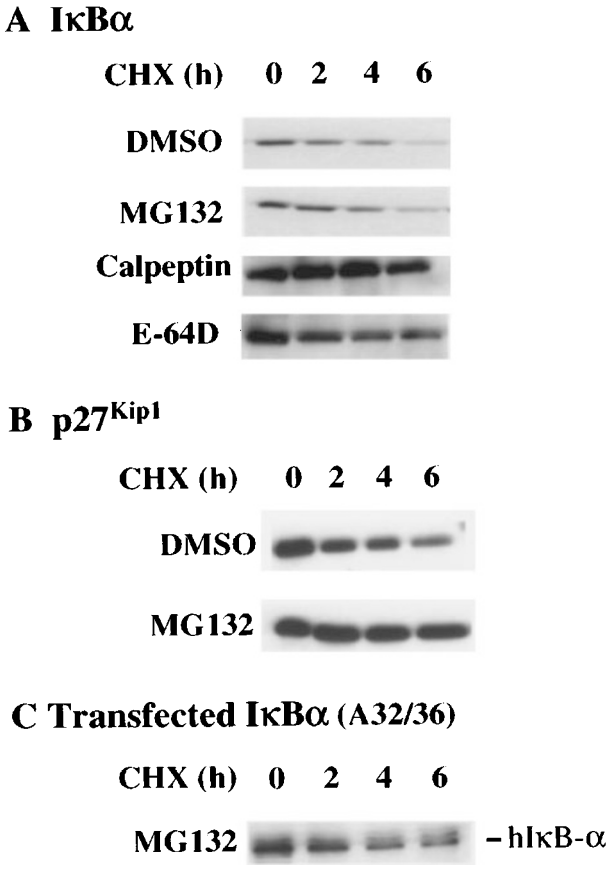

Figure 7 Inhibition of calpain blocks IκBα degradation in NF639 cells. NF639 cells were incubated either with DMSO alone, or with 40 μg/ml MG132, E64-D, or calpeptin for 30 min. Following addition of 50 μg/ml cycloheximide (CHX), WCEs were prepared at the times indicated, and samples (40 μg) subjected to immunoblot analysis for either IκBα (C-21) (**a**) or $p27^{Kip1}$ (M197) (**b**). (**c**) NF639 cells were transfected with 2 μg $pRC_{\beta actin}$ double mutant human IκBα (A32/36) vector. After 36 h, cultures were incubated with 40 μg/ml MG132 for 30 min. Following addition of 50 μg/ml cycloheximide extracts were prepared at the times indicated and subjected to immunoblot analysis using an antibody specific for human IκB-α (C-15). The position of the human IκB-α is as indicated (hIκB-α). A minor slower migrating non-specific band is also seen

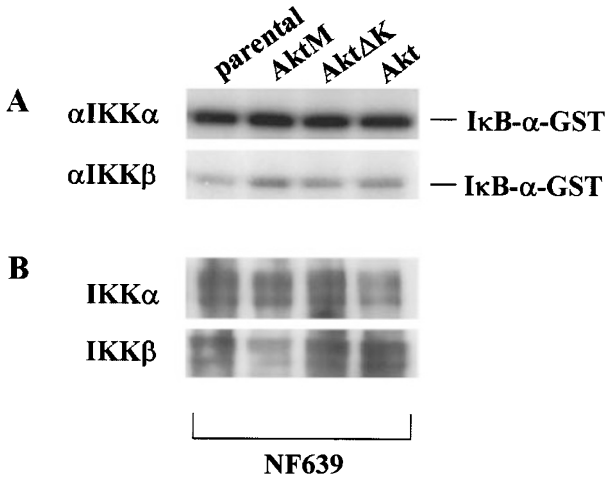

Figure 8 IKK kinase complex is inactive in MMTV-Her-2/neu NF639 cells. (**a**) Total proteins were isolated from NF639 cells, and samples (200 μg) immunoprecipitated with antibodies against either IKKα or IKKβ, as indicated. Two-thirds of the preparations were subjected to a kinase assay using 1 μg IκB-α-GST protein, as substrate. (**b**) The remainders of the preparations were immunoblotted for either IKKα or IKKβ to verify immunoprecipitation

immunoprecipitated with antibodies against either IKKα or IKKβ. The resulting immunoprecipitated proteins were used in a kinase assay with IκB-α-GST as substrate. Expression of AktΔK, which dramatically decreased NF-κB levels above, caused no significant reduction in activity of either IKKα or IKKβ (Figure 8a). In fact, a minor increase of 1.2 ± 0.1-fold was seen in this and a duplicate experiment. Thus, inhibition of Akt does not alter IKK kinase activity in the NF639 cells. Expression of the AktM, which caused a slight increase in NF-κB levels above (Figure 5), resulted in a slight increase in IKK activity (Figure 8a). No difference in IKK activity was noted in the NF639 cells transfected with parental Akt expression vector. Essentially equal amounts of IKK proteins were present in the extracts as verified by immunoblotting (Figure 8b). Thus, activation of the IKKα and IKKβ components of the IKK kinase complex does not appear able to account for the constitutive basal NF-κB binding seen in the MMTV-Her-2/neu NF639 cell line, consistent with the failure of the proteasome inhibitor to substantially affect IκB-α turnover.

Recent work has demonstrated a role for calpain in IκB-α turnover in B cells, which constitutively express NF-κB (Fields *et al*., 2000; Miyamoto *et al*., 1998). Thus, we next investigated the involvement of calpain in IκB-α degradation in the NF639 line using the inhibitors calpeptin and E-64D. While both inhibitors are specific for calpain, the E-64D does not enter cells as well and has been found to be less effective than calpeptin (Fields *et al*., 2000; Miyamoto *et al*., 1998). NF639 cells were incubated in the presence of 40 μg/ml calpeptin or E-64D, or carrier DMSO as control. Calpeptin essentially completely prevented the decay of IκB-α (Figure 7a). Treatment with E-64D also ablated the rate of IκB-α decay, but was somewhat less effective, consistent with the findings of others. Thus, inhibition of calpain ablates IκB-α turnover, indicating the activation of NF-κB that occurs as a result of overexpression of Her-2/neu is mediated, at least in part, by calpain.

Discussion

In this study we show that overexpression of Her-2/neu leads to constitutive induction of NF-κB mediated through a PI3-kinase/Akt kinase pathway that signaled degradation of IκB-α via calpain. The activation of NF-κB could be inhibited by the phosphatase PTEN. Constitutive nuclear NF-κB was observed in Ba/F3 cells overexpressing Her-2/neu + EGFR4, as well as in

cell lines derived from MMTV-Her-2/neu mouse mammary gland tumors and in primary tumor specimens. Inhibition of PI3-kinase by either wortmannin treatment or by ectopic expression of a kinase inactive variant reduced NF-κB levels. Consistent with these findings, negative or positive modulation of Akt kinase activity either repressed or induced NF-κB activity, respectively. Previously, our laboratory demonstrated aberrant NF-κB/Rel activation typifies primary human breast cancers (Sovak *et al*., 1997). Here we begin to provide a mechanism for the aberrant expression of these factors in cancers with overexpression of Her-2/neu. Furthermore, our findings implicate the calcium sensitive cysteine protease calpain family in breast cancer progression through the constitutive activation of NF-κB.

Overexpression of Her-2/neu in breast cancer correlates with a poor prognosis due to enhanced metastatic potential and resistance to chemotherapy (Hortobagyi *et al*., 1999; Slamon *et al*., 1987; Yu *et al*., 1998). The transmembrane receptor encoded by the Her-2/neu oncogene has intrinsic kinase activity even in the absence of ligand that activates receptor-mediated signal transduction. Consistent with these observations, here we detected intrinsic tyrosine activation of Her-2/neu in Ba/F3 overexpressing Her-2/neu + EGFR4, as well as in NF639 cells (data not shown). Recently overexpression of Her-2/neu in immortalized HMECs has been found to result in growth factor independence, acquisition of anchorage-independent growth capacity and ability to induce invasion in a manner similar to breast cancer cells (Ignatoski *et al*., 2000). NF-κB has been implicated in the regulation of a wide spectrum of genes, including those that mediate transformation (e.g., c-*myc*) (La Rosa *et al*., 1994; Kessler *et al*., 1992; Duyao *et al*., 1992; Kim *et al*., 2000) and metastasis (e.g., metalloproteinases) (reviewed in Grimm and Baeuerle, 1993). Thus, it is likely that aberrant NF-κB activity plays an important role in the transformed phenotype displayed in cells with overexpression of Her-2/neu.

Overall our work has implicated NF-κB in the neoplastic development of mammary and liver epithelial tumors (Sovak *et al*., 1997; Arsura *et al*., 2000). We first reported aberrant elevated levels of NF-κB binding in over 85% of mammary tumors in the Sprague-Dawley rat after exposure to 7,12-dimethylbenz(*a*)anthracene (DMBA) (Sovak *et al*., 1997). More recently NF-κB activation was observed within 3 weeks of DMBA treatment when tumors had not yet developed, suggesting that it is an early event in tumor formation (Kim *et al*., 2000). Furthermore, we showed that in Ras- and Raf-transformed rat liver cells, NF-κB levels are enhanced, promoting cell survival, transformed phenotype, and resistance to TGF-β1 treatment (Arsura *et al*., 2000). The induction of NF-κB by Ras was mediated by two pathways involving both Raf and PI3-kinase leading to the activation of the IKK complex (Arsura *et al*., 2000). Importantly, many other tumors have recently been shown to display constitutive activation of NF-κB (reviewed in Rayet and Gelinas, 1999), including the human cutaneous T-cell lymphoma HuT-78 (Giri and Aggarwal, 1998), Hodgkin's lymphomas (Bargou *et al*., 1997), melanoma (Shattuck *et al*., 1994) pancreatic adenocarcinoma (Wang *et al*., 1999), primary adult T-cell leukemia (Mori *et al*., 1999), and head and neck squamous cell carcinoma (Duffey *et al*., 1999). Furthermore, the Tax transforming protein produced by the human T-cell leukemia virus type 1 induces NF-κB activity (Mercurio *et al*., 1997; Sun *et al*., 1994) through activation of both IKK1 and IKK2 (Geleziunas *et al*., 1998; Sumitomo *et al*., 1999b). Moreover, NF-κB induction has been found to inhibit TNF-α-induced cell death of breast, prostate, and bladder cancer cells (Geleziunas *et al*., 1998; Sumitomo *et al*., 1999a) (reviewed in Rayet and Gelinas, 1999). Interestingly, TNF-α activation of NF-κB in primary patient samples overexpressing Her-2/neu was reported to occur via a PI3-kinase/Akt/IKK pathway recently by Zhou *et al*. (2000). Here, we have shown that enhanced basal levels of NF-κB are displayed by specimens of Her-2/neu induced primary mouse breast tumors compared to normal mammary gland, as well as in a cell line derived from one of these tumors. No effect of inhibition of Akt on IKK kinase activity nor substantial involvement of the proteasome in turnover of IκB-α could be detected in the NF639 cell line. Thus, the constitutive activation of NF-κB upon overexpression of Her-2/neu in this mouse mammary tumor cell line appears to be mediated via a non-IKK/proteasome pathway that involves calpain, similar to findings with mature early B cells (Fields *et al*., 2000). The IKK activity seen in the tumor cells by Zhou *et al*. (2000) may have resulted from the *in vitro* exposure to TNF-α. Alternatively, the differences may relate to involvement of different cell types in the two studies. Work is in progress to evaluate the sites of phosphorylation and the role of the PEST domain of IκB-α in its turnover in these cells.

The PTEN lipid phosphatase, which has been shown to modulate PI3-kinase activity and PIP-3 levels, is lost in a variety of tumors (Li *et al*., 1997; Di Cristofano *et al*., 1998; Whang *et al*., 1998). In quiescent untransformed cells, PIP-3 levels are very low, and rapidly increase upon stimulation by growth factors, through activation of PI3-kinase. Accumulation of PIP-3 allows the recruitment and activation of Akt via phosphorylation. Overexpression of PTEN in glioma and breast cancer has been reported to result in the inactivation of Akt and the induction of anoikis, a specific apoptotic pathway initiated by cell detachment from the extracellular matrix (Lu *et al*., 1999). Furthermore, PI3-kinase and Akt regulation of NF-κB has been implicated in survival of cells transformed by Ras (Madrid *et al*., 2000). In our study, ectopic expression of PTEN in the NF639 cells reduced the activity of Akt and the levels of NF-κB binding. Based on our previous work (Sovak *et al*., 1997; Wu *et al*., 1996), this drop in NF-κB likely plays a critical role in induction of apoptosis.

Anti-Her-2/neu antibodies have been shown to be potent growth inhibitors. The human monoclonal

antibody (Herceptin) is currently in use in adjuvant therapy for women with overexpression of Her-2/neu. The systemic administration of the antibodies in combination of cytotoxic chemotherapy in these patients has been shown to increase the length of time of recurrence and the sensitivity to chemotherapy (Ross and Fletcher, 1998). We have then shown that treatment of NF639 cells with anti-neu antibodies decreased NF-κB binding levels, through the inhibition of Akt activation. Thus, it is tempting to speculate that repression of NF-κB is one of the significant targets of these antibodies, and thus agents that inhibit NF-κB may provide additional adjuvant therapy reagents in the treatment of breast disease.

Materials and methods

Cell growth and treatment conditions

MCF-10F human mammary epithelial cells (HMECs) were established from a patient with fibrocystic disease and do not display characteristics of a malignant phenotype. They represent a non-tumorigenic, immortally transformed cell line (Calaf and Russo, 1993), and were cultured as we have published previously (Sovak *et al*., 1999). The MMTV-c-neu transgene cell line NF639, derived from mammary gland tumors (kindly provided by P Leder, Harvard Medical School, Boston, MA, USA), was cultured as described previously (Elson and Leder, 1995). Ba/F3 cells transfected to express EGFR-1 (Ba/F3-1), Her-2/neu or EGFR-2 (Ba/F3-2), EGFR-1 and Her-2/neu (Ba/F3-1 + 2), and Her-2/neu and EGFR-4 (Ba/F3-2 + 4) were kindly provided by David Stern (Yale University, New Haven, CN, USA) (Riese *et al*., 1996). The parental cells and isolated clones were grown in RPMI supplemented with 10% fetal bovine serum, conditioned medium from WEHI 3 cells, and antibiotics, as described previously (Riese *et al*., 1996). Where indicated cells were treated with the PI3-kinase inhibitor 100 nM wortmannin (Sigma Chemical Co., St Louis, MO, USA) in DMSO or DMSO alone as control, or with 1 μg anti-neu antibody (Ab-4, Calbiochem, San Diego, CA, USA). Alternatively, the proteasome inhibitor MG132, or calpain inhibitor calpeptin (Calbiochem), or E-64D (Peptide International) dissolved in DMSO, or a similar dilution of DMSO as control, was added. For half-life studies, 50 μg/ml cycloheximide was added, and whole cellular extracts prepared and subjected to immunoblot analysis, as described below.

Electrophoretic mobility shift analysis

Nuclear extracts were prepared from breast cancer cells by a modification of the method of Dignam *et al*. (1983). Briefly, cells were washed twice with ice cold PBS (Ca^{2+} and Mg^{2+} free) containing protease inhibitors (0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 ug/ml leupeptin (LP)). They were then resuspended in 1 ml of cold hypotonic RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.4) containing 0.5% NP-40 detergent plus protease inhibitors as above. Following a 10 min incubation on ice, the cells were Dounce homogenized until cell lysis occurred. Nuclei were resuspended in two packed nuclear volumes of extraction buffer C (420 mM KCl, 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) plus protease inhibitors as above, and incubated on ice for 30 min. Protein concentration was determined using the Bio-Rad protein assay, following the manufacturer's directions (Bio-Rad Laboratories, Hercules, CA, USA). The sequence of the URE NF-κB-containing oligonucleotide from the c-*myc* gene (Duyao *et al*., 1990) is as follows: 5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′, where the underlined region indicates the core binding element. The mutant URE has a two G to C base pair conversion, indicated in bold, which blocks NF-κB/Rel binding: 5′-GATCCAAGTCCGCCTTTTCCCCAACC-3′. The Octomer-1 (Oct-1) oligonucleotide has the following sequence: 5′-TGTCGAATGCAAATCACTAGAA-3′. The sequence of the Sp1 oligonucleotide is 5′-ATTCGATCGGGGCGGGGC-GACC-3′. The sequences of the PU.1- and TCF-1-containing oligonucleotides are as follows, PU.1 : 5′-GATC-TACTTCTGCTTTTG-3′; TCF-1 : 5′-GGGAGACTGAGAA-CAAAGCGCTCTCACAC-3′ (van de Wetering *et al*., 1991). Oligonucleotides were end labeled with large Klenow fragment of DNA polymerase and [^{32}P]dNTPs. The electrophoretic mobility shift assay (EMSA) was performed using approximately 4 ng of labeled oligonucleotide (40 000 d.p.m.), 5 μg of nuclear extract, 5 μl of sample buffer (10 mM HEPES pH 7.5, 4 mM DTT, 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 1 × TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0). For antibody supershift analysis, the binding reaction was performed in the absence of the probe, the appropriate antibody was added and the mixture incubated for 16 h at 4°C. The probe was then added and the reaction incubated an additional 30 min at 25°C and the complexes resolved by gel electrophoresis, as above. Antibodies used include: anti-RelA subunit: sc-372X, anti-p50 subunit: sc-114, anti-p52 subunit: sc-7386, and anti-c-Rel subunit: sc-71 (all from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). For the IκB-α blocking assays, 1 μg of IκB-α-glutathione-S-transferase (GST) fusion protein was added to the binding reaction, as described for the antibody supershift analysis.

Transfection analysis

Twenty-four hours after plating at 30% confluence in P100 dishes, NF639 cells were incubated with 8 μg DNA in 10 μl FuGENE Reagent, according to manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). After 24 or 48 h, cells were harvested, nuclear fraction isolated and subject to EMSA analysis as described above. The SR-αΔp85 (dead kinase) PI3-kinase construct has been described elsewhere (Kotani *et al*., 1994). AKT, AKTΔK (dead kinase), and AktM (myristylated membrane kinase) were cloned in a cytomegalovirus (CMV) promoter-driven expression vector and were kindly provided by Z Luo (Boston Medical School, Boston, MA, USA). The PTEN plasmid was cloned in pCMV5, and was kindly provided by JE Dixon (University of Michigan, Ann Arbor, MI, USA). Wild-type (E8) and mutant (mutE8) NF-κB element-thymidine kinase (TK) promoter-chloramphenicol acetyl-transferase (CAT) reporter vectors were constructed as reported previously (Duyao *et al*., 1990). Briefly, these consisted of two copies of either the wild-type or mutant NF-κB element from upstream of the c-*myc* promoter, sequences given above. Twenty-four hours after plating at 30% confluence in P35 dishes, NF639 cells were transfected, in triplicate, with 4 μg DNA in 4 μl FuGENE Reagent. After 24 h, cells were harvested and equal amounts of protein

subjected to CAT assays, as we have described previously (Sovak *et al*., 1997). Alternatively, cells were co-transfected with an NF-κB-element driven luciferase construct (Rawadi *et al*., 1999), kindly provided by Georges Rawadi (Hoescht-Marion-Roussel, Romainville, France) in the presence of parental or Akt expression vectors and luciferase activity measured as described previously (Dolwick *et al*., 1993). Standard deviation was obtained using the Student's *t*-test. The $pRC_{\beta actin}$ containing double mutant human IκB-α (A32/36) was kindly provided by Michael Karin (University of California, San Diego, CA, USA).

Immunoblot analysis

Ba/F3 cells were rinsed with cold PBS, and harvested in lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 150 mM NaCl, 0.5 mM DTT, 2 μg/ml aprotinin, 2 μg/ml LP, 0.5 mM PMSF, 0.5% NP40). Whole cell extracts (WCEs) were obtained by sonication, followed by centrifugation at 14 000 r.p.m. for 30 min. WCEs, in the amount indicated in each experiment, were subjected to electrophoresis in a 10% polyacrylamide-SDS gel, transferred to PVDF membrane (Millipore, Bedford, MA, USA) and immunoblotting, as previously described (Arsura *et al*., 1997). Antibodies used were: C-21, mouse IκB-α; C-15, human-specific IκB-α; M197, p27Kip1 (all from Santa Cruz Biotechnology).

Akt kinase assay

The Akt kinase assay, was performed following the directions of Akt Kinase Assay Kit (#9840, New England Biolabs, Beverly, MA, USA). Briefly samples (80 μg) of WCEs were immunoprecipitated with an agarose conjugated anti-Akt antibody (#9279, New England Biolabs) at 4°C overnight. The immunoprecipitate was resuspended in kinase buffer and the assay performed at 30°C for 45 min, using 1 μg of GSK3α-GST fusion protein as substrate in the presence of 10 μM ATP. The resulting products were resolved in a 10% polyacrylamide-SDS gel and subjected to immunoblotting, as above, using phosphospecific GSK-3α antibody (#9331, New England Biolabs).

Isolation of MMTV-c-neu mammary gland tumor and normal mammary gland proteins

MMTV-neu tumors and histologically normal mammary gland tissue surrounding the tumor (Muller *et al*., 1996) were frozen in liquid nitrogen, and powdered using mortar and pestle. For isolation of nuclear proteins, the powders (1 gm tissue/ml) were resuspended in homogenization buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml LP, 5 μg/ml aprotinin), and homogenized with a Dounce. The concentration of KCl was then adjusted to 100 mM and the samples were centrifuged at 4000 r.p.m. for 15 min at 4°C. The pellets were then resuspended in 100 μl extraction buffer (10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml LP, 5 μg/ml aprotinin), and incubated at 4°C for 30 min with gentle agitation. The samples were then centrifuged at 15 000 r.p.m. at 4°C for 15 min, and the supernatant (nuclear extract) recovered.

Immunoprecipitation and IKK kinase assay

NF639 cells were washed with cold PBS and WCEs prepared in PD buffer (40 mM Tris pH 8.0, 500 mM NaCl, 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml LP, 1 μg/ml pepstatin, 1 mM DTT, 0.1% NP-40). Samples (200 μg) were precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at 4°C. The precleared samples were subjected to immunoprecipitation in 500 μl PD buffer. Antibody (1 μl) against either IKKα (Sc7182) or IKKβ (Sc7607, both from Santa Cruz Biotechnology Inc) was added to the mixture and the reaction incubated overnight at 4°C with gentle agitation. The following day, 100 μl protein A-Sepharose beads were added and the samples incubated for 1 h at 4°C. The samples were then washed three times in PD buffer. Two-thirds of the immunoprecipitate were subjected to a kinase assay essentially as published previously (Mercurio *et al*., 1997). Briefly, samples were resuspended in 20 μl of kinase buffer C [20 mM HEPES pH 7.7, 2 mM $MgCl_2$, 10 μM ATP, 3 μCi [$\gamma-^{32}$P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml LP, 1 μg/ml pepstatin, 1 mM DTT], and incubated at 30°C for 45 min in the presence of 200 ng IκB-α-GST fusion protein, as substrate. The kinase reaction was stopped by addition of 2 × SDS-polyacrylamide gel electrophoresis sample buffer, subjected to polyacrylamide-SDS gel analysis and visualized by autoradiography. The remaining immunoprecipitated fraction was subjected to immunoblot analysis, as described above.

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