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Negative Regulation of Transactivation Function but Not DNA Binding of NF-κB and AP-1 by IκBβ1 in Breast Cancer Cells*

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The transcription factor NF-κB regulates the expression of genes involved in cancer cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. In normal cells NF-κB is maintained in the cytoplasm by protein-protein interaction with inhibitor IκBs. In contrast, in cancer cells a substantial amount of NF-κB is in the nucleus and constitutively activates target genes. To understand the mechanisms of constitutive NF-κB activation, we have analyzed the function of IκBα and IκBβ in breast cancer cells. In most cases, constitutive NF-κB DNA binding correlated with reduced levels of either IκBα or IκBβ isoforms. Overexpression of IκBα but not IκBβ1 resulted in reduced constitutive DNA binding of NF-κB in MDA-MB-231 cells. Unexpectedly, IκBβ1 overexpression moderately increased 12-*O*-tetradecanoylphorbol-13-acetate- and interleukin-1-inducible NF-κB DNA binding. 12-*O*-Tetradecanoylphorbol-13-acetate- and interleukin-1-induced transactivation by NF-κB, however, was lower in IκBβ1-overexpressing cells. Mutants of IκBβ1 lacking the C-terminal casein kinase II phosphorylation sites, which form a stable complex with DNA bound NF-κB without inhibiting its transactivation in other cell types, repressed the transactivation by NF-κB in MDA-MB-231 cells. Consistent with the results of transient transfections, the expression of urokinase plasminogen activator, an NF-κB target gene, was reduced in IκBβ1-overexpressing cells. These results suggest that depending on the cell type, IκBβ1 represses the expression of NF-κB-regulated genes by inhibiting either DNA binding or transactivation function of NF-κB.

Regulation of gene expression by the NF-κB[1]/Rel family of transcription factors is controlled mainly by the inhibitory IκB proteins which include IκBα, IκBβ, IκBγ, and IκBε (1–4). The active complex of NF-κB is composed of homodimers and heterodimers of p50, RelA, RelB, and c-Rel. These complexes are sequestered in the cytoplasm by IκBs. Extracellular signal-induced phosphorylation and subsequent degradation of IκBs is essential for nuclear translocation of NF-κB. NF-κB binds to the recognition elements in the promoter region of target genes and activates transcription. Transactivation by NF-κB involves interaction with transcriptional coactivators such as CBP/p300 and SRC-1 and general transcription factors including TBP (5–8). In addition, NF-κB interacts with transcription factors such as activator protein 1 (AP-1) and serum response factors and synergistically activates transcription (9, 10).

IκB proteins determine the duration of transactivation by NF-κB. IκBα is involved in transient activation of NF-κB because it is degraded rapidly upon stimulation and is resynthesized by activated NF-κB (1–4). Most of the inducers of NF-κB cause degradation of IκBα. The newly synthesized IκBα sequesters NF-κB in the cytoplasm and terminates the signal. IκBβ is involved in persistent activation of NF-κB (11). Degradation of IκBβ is much more delayed than IκBα and resynthesis is independent of NF-κB (11). Degradation of IκBβ is dependent on the cell type and the extracellular signals that induce NF-κB. For example, whereas interleukin 1 (IL-1) induces degradation of IκBβ in most cell types tested, tumor necrosis factor (TNF-α) induces degradation of IκBβ in E29.1 T cell hybridomas but not in Jurkat cells (11, 12). Together, but not separately, TNF-α and interferon γ induce degradation of IκBβ in PC12 cells (13). The ability of IκBβ to repress NF-κB DNA binding is determined by the basal phosphorylation of the C-terminal PEST domain (14). The newly synthesized hypophosphorylated IκBβ functions as a chaperone for NF-κB by protecting it from IκBα (15). The IκBβ·NF-κB complex enters the nucleus, binds to DNA, and activates transcription (16, 17). The phosphorylation of the PEST domain by casein kinase II or the association of the IκBβ·NF-κB complex with high mobility group I (HMG I) proteins on selected promoters converts IκBβ to a repressor of NF-κB DNA binding activity (14, 17).

Two isoforms of IκBβ, β1 and β2, have been described recently (18). These isoforms differ in their C-terminal amino acids. IκBβ1 is functionally similar to IκBα and efficiently represses the activity of p50:RelA heterodimers. It is found in both the nucleus and cytoplasm and is degraded by various stimuli. In contrast, IκBβ2 is a cytoplasmic protein that is refractory to signal-induced degradation. Although both isoforms are indistinguishable in their binding preferences to cellular NF-κB/Rel homo- and heterodimers, IκBβ2 is more effective in inhibiting RelA homodimers than p50:RelA and p50:c-Rel heterodimers (18). This property of IκBβ2 is similar to that of IκBε which preferentially inhibits the activity of RelA homodimers (19).

There is increasing evidence for the role of NF-κB in cancer progression and resistance to chemotherapy (20, 21). Constitutive activation of NF-κB has been observed in Hodgkin's lymphoma, melanomas, juvenile myelomonocytic leukemia, cuta-

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[1] The abbreviations used are: NF-κB, nuclear factor κB; AP-1, activator protein 1; IκB, inhibitor κB; IL-1, interleukin 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; uPA, urokinase plasminogen activator; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; MEM, minimum Eagle's medium; EGF, epidermal growth factor; HMG I, high mobility group I; ERα, estrogen receptor α.

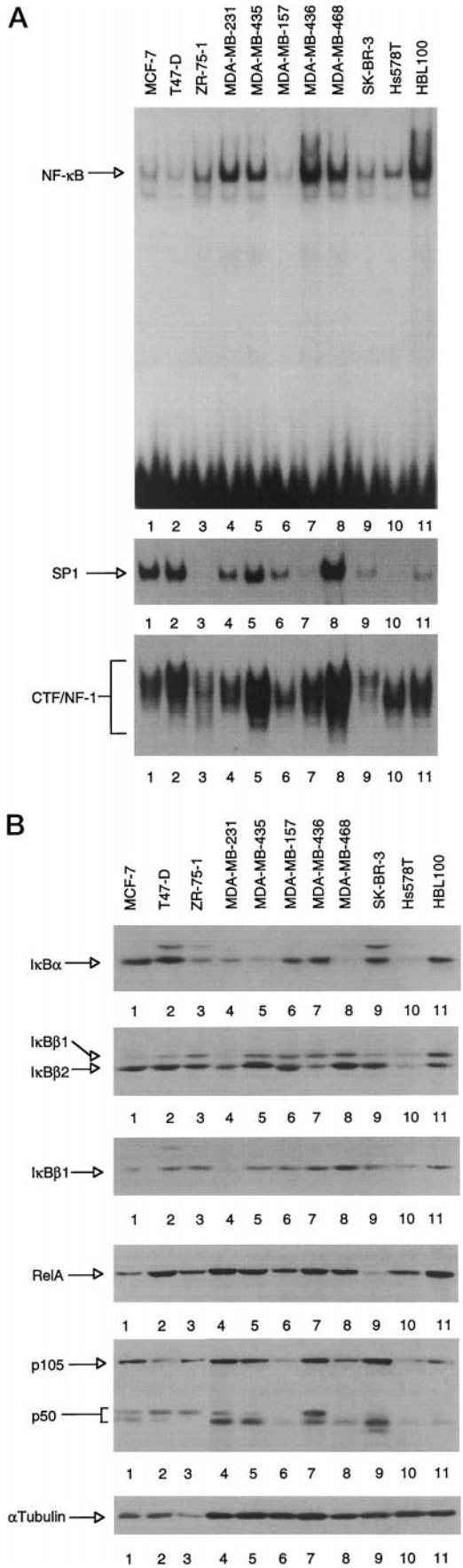

FIG. 1. **Differential expression of IκBα, IκBβ1, and IκBβ2 in breast cancer cells.** *A,* DNA binding activity of NF-κB in breast cancer cell lines. Whole cell extracts (6 μg) were incubated with double-stranded oligonucleotide containing NF-κB, SP1, or CTF/NF-1-binding sites and subjected to EMSA. CTF/NF-1 displays multiple bands due to alternative splicing. *B,* RelA and IκB expression in breast cancer cell lines. Whole cell extracts (50 μg) from indicated cell lines were analyzed for RelA, p105/p50, and IκBs by Western blotting. For identification of IκBβ1, an antibody raised against the unique C-terminal region of IκBβ1 was used in Western blotting. Integrity of proteins in each lanes was verified by reprobing the blot with antibody against α-tubulin.

neous T cell lymphoma, and human T cell leukemia virus type I Tax protein-mediated cellular transformation (22–26). We and others (27, 28) have shown that NF-κB is constitutively active in a subset of breast cancers. In this study, we have investigated the role of IκBα and IκBβ in constitutive activation of NF-κB in breast cancer cells. Constitutive DNA binding correlated with reduced levels of either IκBα or IκBβ. Both isoforms of IκBβ are present in breast cancer cell lines and primary breast cancers. Overexpressed IκBα but not IκBβ1 repressed constitutive DNA binding of NF-κB in MDA-MB-231 cells. IκBβ1, in contrast, repressed transactivation by NF-κB. Unlike in the mouse embryonal carcinoma cell line P19 and the human embryonic kidney cell line 293, mutants of IκBβ1 that cannot be phosphorylated by casein kinase II efficiently repressed transactivation by NF-κB in MDA-MB-231 cells (17, 29). Taken together, these results reveal a novel cell type-specific inhibitory regulation of transactivation function but not DNA binding of NF-κB by IκBβ1.

EXPERIMENTAL PROCEDURES

Cell Culture—All breast cancer cell lines were purchased from ATCC, and their growth conditions have been described previously (30). 10^{-9} M insulin was added to MDA-MB-231 and its clones during long term culturing.

Recombinant Plasmids—NF-κB/CAT reporter gene, human IκBα, and mouse IκBβ expression vectors have been described previously (27). The AP-1/CAT reporter was a generous gift of E. O'Neill (Merck) and contains three copies of an AP-1 site from the human metallothionein gene (31). Human IκBβ and its mutants were generous gifts from D. Ballard (Vanderbilt University, Nashville, TN) and were described previously (29). mIκBβ1ΔC, which lacks 50 amino acids at the C-terminal, was generated by cloning a *Not*I-*Hin*dIII fragment of mIκBβ1 to the pcDNA3 expression vector (Invitrogen). Retrovirus constructs containing hIκBα and mIκBβ1 coding sequences were prepared by cloning cDNAs into the respective *Eco*RI and *Not*I sites of the modified retrovirus vector LxSN (32).

Retrovirus Preparation and Transduction—LxSN vectors were amphotrophically packaged into the AM12 packaging cell line (33), and the cell-free supernatant was used to transduce MDA-MB-231 cells as described previously (34). Vector containing clones were isolated by growing cells in G418 (1 mg/ml).

Electrophoretic Mobility Shift Assay (EMSA)—Whole cell extracts from cells were prepared and subjected to EMSA as described previously (27). Densitometric analysis was performed to classify constitutive DNA binding activity.

Western Blotting—Cell extracts were prepared in radioimmunoprecipitation assay buffer (35) and subjected to Western blotting as recommended by the antibody manufacturers (Santa Cruz Biotechnology and Sigma). For quantitation, autoradiograms were scanned using a densitometer.

DNA Immunoprecipitation—Nuclear extracts were prepared as described previously (36). Nuclear extracts supplemented with 1% Nonidet P-40 were precleared in insoluble protein A solution (Sigma) and subjected to EMSA reaction. Antibodies (0.2 μg) were added, and incubation was carried out for 1 h on ice. Protein A-agarose (20-μl bead volume) was added, and the reaction mixture was incubated for 1 h at 4 °C with gentle rocking. Protein A-agarose was collected by centrifugation, washed five times in 1× EMSA buffer (10 mM Tris-HCl, 0.5 mM EDTA, 100 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40), and subjected to SDS-polyacrylamide gel electrophoresis. The amount of radioactive DNA immunoprecipitated with the antibody complex was visualized by autoradiography and quantitated by densitometric scanning.

Transient Transfections and CAT Assays—Transient transfections and the CAT assay have been described previously (27). Statistical analysis was performed using Statview software (version 4.1; Abacus

TABLE I
Summary of NF-κB DNA binding activity and IκB expression in breast cancer cell lines
ERα status in these cell lines has been described previously (30, 37). A value of 4+ is assigned to the most intense signal among various cell lines.

	ERα	NF-κB	IκBα	IκBβ1	IκBβ2
MCF-7	+	+	++++	+	+++
T47-D	+	+	++++	++	+++
ZR-75	+	++	++	++	+++
MDA-MB-231	−	+++	++	+	+
MDA-MB-435	−	+++	+	++	++++
MDA-MB-157	−	+	+++	++	+++
MDA-MB-436	−	++++	+++	+++	+
MDA-MB-468	−	+++	+	++++	++++
SK-BR-3	−	++	++++	++	+++
Hs578T	−	++	+	++	+
HBL100	−	++++	++++	+++	+++

Concepts, Berkeley, CA). Data were evaluated by one-factor analysis of variance and Fisher's protected least significant difference as a post hoc test.

Serum and Epidermal Growth Factor (EGF) Treatment and Northern Blotting—For serum stimulation, cells were maintained in MEM without serum for 48 h. MEM with 10% fetal calf serum was added, and the cells were harvested after 4 h. For EGF treatment, cells were maintained in MEM (without fetal calf serum) for 48 h. EGF (30 ng/ml) was added, and cells were harvested after 4 h. RNA was prepared by the guanidinium isothiocyanate/cesium chloride method and subjected to Northern blotting as described previously (30).

RESULTS

Differential Expression of IκBs in Breast Cancer Cells—Constitutive NF-κB DNA binding activity in several breast cancer cell lines was compared with the expression level of IκBα and IκBβ (Fig. 1 and Table I). MCF-7, T47-D, and MDA-MB-157 cells displayed the least amount of NF-κB DNA binding and higher levels of IκBα and IκBβ compared with other cell lines (see "Experimental Procedures"). In comparison, NF-κB DNA binding activity in other cell lines were either high (MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-468, and HBL-100) or intermediate (ZR-75–1, SK-BR-3, and Hs578T) (Fig. 1*A* and Table I). Among these cell lines, MCF-7, T47-D, and ZR-75–1 express estrogen receptor α (ERα) and are more differentiated than other cell lines (37). Most of the cell lines that displayed elevated NF-κB DNA binding activity are ERα-negative, consistent with our previous report (27). In contrast to NF-κB, DNA binding of SP1 and CTF/NF-1 family transcription factors did not correlate with ERα status (Fig. 1*A*). The NF-κB complex is a heterodimer of RelA and p50 subunits as determined by antibody supershift assays (data not shown). In general, NF-κB DNA binding activity correlated inversely with the levels of IκBα, IκBβ1, and IκBβ2 (Fig. 1*B*, Table I). For example, higher NF-κB DNA binding correlated with reduced levels of either IκBα (MDA-MB-435 and MDA-MB-468), IκBβ2 (MDA-MB-436) or IκBα, or IκBβ1 and IκBβ2 (MDA-MB-231). IκBβ2 is the major IκBβ in breast cancer cell lines with the exception of HBL-100 and MDA-MB-436 cells which contained similar levels of IκBβ1 and IκBβ2. Reverse transcription-polymerase chain reaction was used to confirm the presence of transcripts corresponding to IκBβ1 and IκBβ2 in breast cancer cells (data not shown). Proteins corresponding to IκBβ1 and IκBβ2 were also observed in primary breast cancers (data not shown). There was no correlation between IκB expression and NF-κB DNA binding activity in Hs578T and HBL-100 cells. Despite reduced levels of both IκBα and IκBβ2, NF-κB DNA binding was intermediate in Hs 578T. It is possible that this cell line overexpresses other IκB proteins. HBL-100 cells displayed elevated NF-κB DNA binding activity despite the presence of IκBα and IκBβ2. Whether elevated NF-κB DNA binding in this cell line is due to 1:1 ratio of IκBβ1 and IκBβ2 or due to reduced levels of other IκB proteins is not known. Constitutive NF-κB DNA binding activity did not correlate with elevated levels of RelA and p50 proteins (Fig. 1*B*).

The Effect of IκBα and IκBβ1 Overexpression on Constitutive NF-κB DNA Activity in MDA-MB-231 Cells—To determine the role of IκBα and IκBβ in constitutive NF-κB activation, we generated MDA-MB-231 cells overexpressing IκBα and IκBβ1. The levels of IκBα and IκBβ1 in cells transduced with empty retrovirus (LxSN3), IκBα coding retrovirus (IκBα3–7), and IκBβ1 containing retrovirus (IκBβ21–25) were determined by Western blotting (Fig. 2*A*). Two clones containing vector alone (called LxSN3 and LxSN5 cells hereafter), two clones with IκBα (IκBα5 and IκBα7 cells), and three clones with IκBβ1 (IκBβ21, IκBβ22, and IκBβ23) were chosen arbitrarily for this study. NF-κB DNA binding activity in these clones is shown in Fig. 2*B*. IκBα overexpression resulted in reduced constitutive DNA binding (compare *lane 1* and *2* with *lanes 3* and *4*). Unexpectedly, constitutive NF-κB DNA binding was the same in IκBβ cells and LxSN cells (compare *lanes 1* and *2* with *lanes 5–7*). Similar results were obtained when nuclear extracts instead of whole cell extracts were used (Fig. 2*B, lanes 8–14*). Also, a similar binding pattern was obtained with PRDII element (NF-κB-binding site in the interferon-β gene promoter which is flanked by the HMG I protein-binding site) and the NF-κB-binding site of urokinase plasminogen activator (uPA) promoter (data not shown). The DNA·NF-κB complex is a heterodimer of p50 and RelA as antibodies against both proteins supershifted the complex (data not shown). IκBα or IκBβ overexpression did not affect DNA binding of AP-1 (Fig. 2*B, lower panel*).

As stated earlier, the hypophosphorylated IκBβ1 enters the nucleus and forms a stable complex with DNA-bound NF-κB. Inability of IκBβ1 to reduce NF-κB DNA binding activity could be due to unimpaired interaction of the IκBβ1·NF-κB complex with DNA. To address this possibility, we performed DNA immunoprecipitation assays with nuclear extracts from LxSN3, IκBα5, and IκBβ21 cells. Radiolabeled DNA·NF-κB complexes were immunoprecipitated with either RelA antibody, IκBα antibody, or IκBβ antibody. Antibody against *neu* oncogene was used as a negative control. DNA·NF-κB complex was efficiently immunoprecipitated by RelA antibody (Fig. *2C, lanes 1, 5,* and *9*). The amount of DNA·NF-κB complex immunoprecipitated by RelA antibody was similar to the amount of NF-κB·DNA complex identified in EMSA. Neither IκBα antibody nor *neu* antibody immunoprecipitated DNA·NF-κB complex from nuclear extracts (*lanes 2, 4, 6, 8, 10,* and *12*). IκBβ antibody immunoprecipitated radiolabeled probe from only IκBβ21 cells (compare *lanes 3, 7,* and *11*). These results indicate that IκBβ1 either binds directly to the NF-κB-binding site or forms a stable complex with DNA-bound NF-κB. Whatever the mechanism involved, DNA binding of IκBβ1 did not interfere in DNA-NF-κB interaction. IκBβ1-DNA interaction was

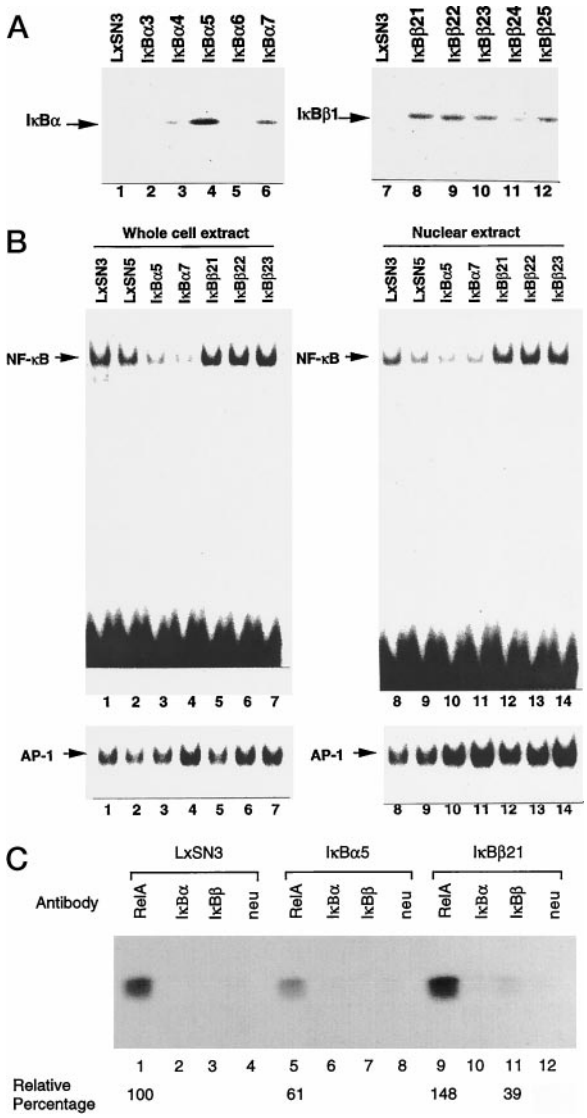

FIG. 2. **Overexpression of hIκBα and mIκBβ1 in MDA-MB-231 cells.** *A,* IκBα and IκBβ1 in transduced clones. Whole cell extracts from individual G418-resistant clones of MDA-MB-231 cells were subjected to Western blotting using indicated antibodies. *B,* NF-κB DNA binding activity in IκBα- and IκBβ1-overexpressing cells. Whole cell extracts (6 μg, *lanes 1–7*) or nuclear extracts (10 μg, *lanes 8–14*) from vector alone (*LxSN3* and *LxSN5*), IκBα-overexpressing (*IκBα5* and *IκBα7*), or IκBβ1-overexpressing (*IκBβ21, IκBβ22,* and *IκBβ23*) cells were subjected to EMSA with either NF-κB probe (*top panel*) or AP-1 probe (*bottom panel*). *C,* IκBβ1 is associated with NF-κB-binding site containing probe. An EMSA reaction with radiolabeled NF-κB probe was performed with 15 μg of nuclear extracts from indicated clones. EMSA reactions were subjected to immunoprecipitation using indicated antibodies. Antibodies against RelA and *neu* served as positive and negative controls, respectively. The amount of radioactive probe precipitated by RelA antibody from LxSN3 cells was set as 100%, and the relative difference is indicated.

not observed in antibody supershift assays possibly due to the unstable nature of the complex under electrophoresis conditions (data not shown).

Inducible DNA Binding of NF-κB in Cells Overexpressing IκBα and IκBβ—To investigate the effect of IκBα and IκBβ on inducible DNA binding of NF-κB, we performed EMSA with extracts from cells treated with either TPA or IL-1β for 1 h. Inducible NF-κB DNA binding activity was lower in IκBα cells compared with other cells (Fig. 3*A*). As with constitutive DNA binding, IκBβ failed to inhibit inducible NF-κB DNA binding. In fact, we consistently observed higher DNA binding in TPA-treated IκBβ cells compared with other cells (compare *lanes 2, 5, 14, 17,* and *20*). IκBα and IκBβ overexpression did not affect DNA binding of AP-1 (Fig. 3*A, lower panel*).

To determine whether IκBα and IκBβ overexpression alters the time course of NF-κB activation, EMSA was performed with the extracts from cells treated with TPA and IL-1 for specific intervals. TPA-inducible DNA binding activity was observed after 60 min of stimulation in all three cell types (Fig. 3*B*, compare *lanes 4, 13*, and *22*) and started to decline after 3 h of stimulation. We consistently observed increased NF-κB DNA binding activity in TPA-treated IκBβ cells compared with other cells. There was no cell type-specific variation in the rate of IκBα degradation. Approximately 50% of IκBα was present at 60 min and 2 h after TPA addition, and its level increased at 3 h (Fig. 3*B*). NF-κB DNA binding activity at 6, 9, and 24 h after TPA addition was higher than in untreated cells which correlated with reduced levels of IκBα. Degradation of IκBβ1 was minimal (<25%) which was evident at 2 and 3 h post-stimulation. We next examined the time course of IL-1-induced NF-κB DNA binding activity (Fig. 3*C*). NF-κB DNA binding increased within 30 min of IL-1 addition in all three cell types. There was a difference in the termination of inducible DNA binding. In LxSN5 and IκBα5 cells, maximum DNA binding was observed after 1 h of IL-1 addition and returned almost to basal levels by 2 h. In contrast, in IκBβ21 cells, a substantial level of inducible DNA binding persisted after 2 h of IL-1 addition. Similar results were obtained with three other IκBβ-overexpressing clones (data not shown). As with TPA-treated cells, IκBα degradation occurred at a similar rate in all three cell types, and partial degradation of IκBβ (<30%) coincided with induction of NF-κB DNA binding in IκBβ21 cells (data not shown). Taken together, our results demonstrate that although IκBα did inhibit constitutive DNA binding, neither IκBα nor IκBβ1 had much effect on inducible DNA binding of NF-κB in MDA-MB-231 cells.

Activity of NF-κB- and AP-1-dependent Promoters in IκBα- and IκBβ1-overexpressing Cells—The inability of IκBβ1 to repress constitutive DNA binding of NF-κB was unexpected. However, it is possible that IκBβ1 may convert the NF-κB·DNA complex to a transcriptionally inactive complex similar to retinoblastoma protein-induced inactivation of the NF-κB·DNA complex (38). To address this possibility, we performed transient transfection experiments using an NF-κB-dependent CAT reporter gene (NF-κB/CAT, see Ref. 27). Expectedly, constitutive and inducible activities of the NF-κB/CAT were lower in IκBα cells compared with LxSN cells (Fig. 4*A*). Interestingly, inducible activities of NF-κB/CAT, particularly with IL-1, were lower in IκBβ cells. The NF-κB/CAT reporter gene expression requires the binding site for NF-κB because a similar reporter in which the NF-κB-binding site has been mutated is inactive in MDA-MB-231 cells (27).

As stated in the Introduction, NF-κB interacts with AP-1, and this interaction results in synergistic activation of transcription. Because IκBβ1 inhibited NF-κB-dependent gene expression despite efficient DNA binding, it is possible that IκBβ1 in the NF-κB·DNA complex prevents synergistic interaction with AP-1 either due to a conformational change or steric hindrance. We measured constitutive and TPA-inducible activity of AP-1/CAT reporter gene in all cell lines. Similar levels of constitutive and TPA-inducible activity were observed in LxSN5, IκBα5, and IκBα7 cells (Fig. 4*B*). Due to differences in cell passage number, we consistently observed lower constitutive and TPA-inducible activities in LxSN3 cells. These cells were passed ~15 more times than other clones. For unknown reasons, decreasing levels of AP-1 transcriptional activation with increasing cell passage were consistently observed with this cell line. The constitutive and TPA-inducible activities of

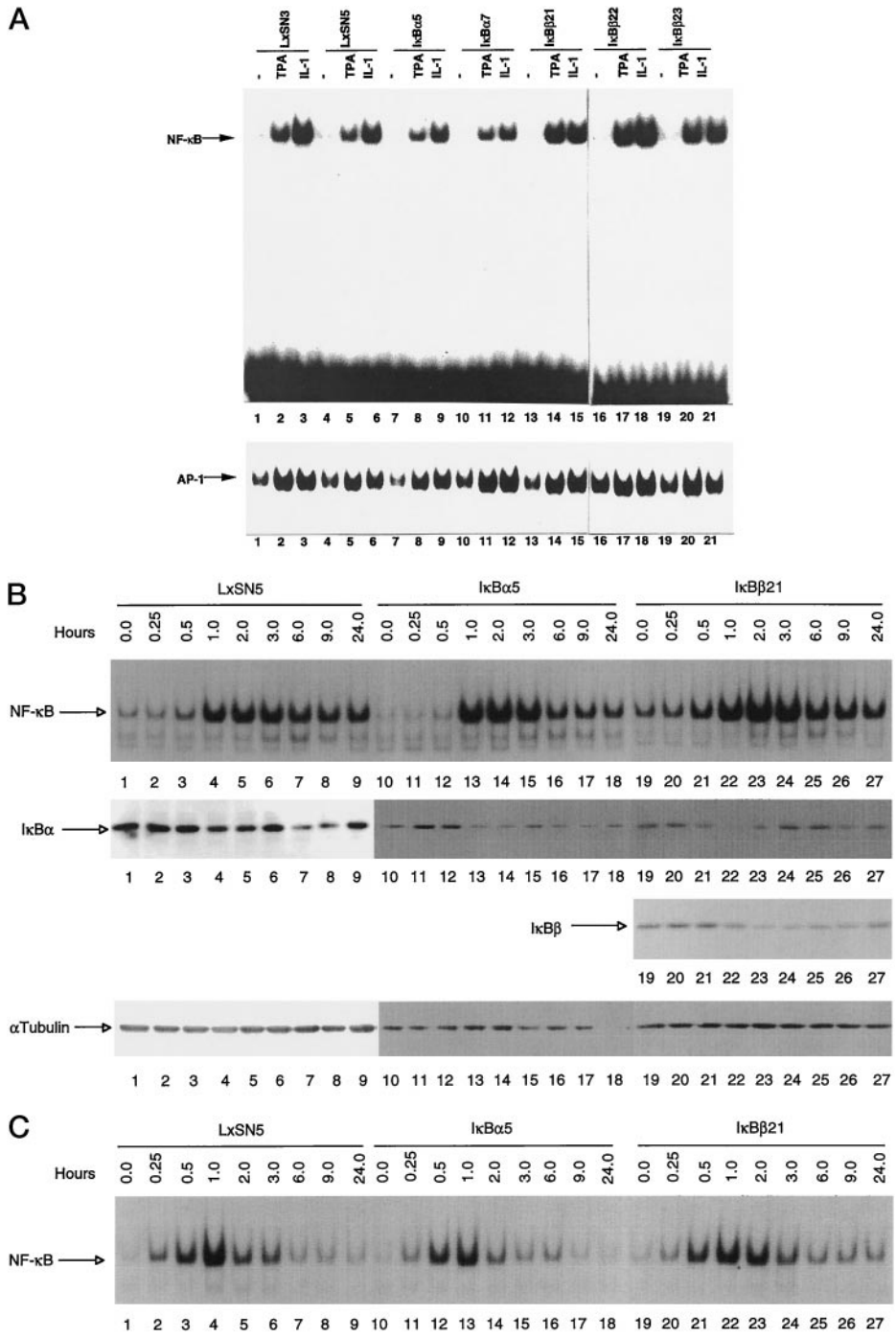

FIG. 3. **Inducible DNA binding of NF-κB in IκB-overexpressing cells.** *A,* TPA- and IL-1β-inducible DNA binding activity. Whole cell extracts from untreated (−), TPA-treated (125 nM), or IL-1β-treated (2.5 ng/ml) cells were subjected to EMSA with either NF-κB probe (*top panel*) or AP-1 probe (*bottom panel*). *B,* time course of TPA-inducible NF-κB DNA binding activity. Whole cell extracts were prepared at specific intervals after treatment with TPA and subjected to EMSA (*top panel*). IκBα in all cell types and IκBβ1 in IκBβ21 cells were analyzed by Western blotting. Western blot with LxSN5 cell extracts was exposed three times longer than other blots, and it was performed with double the amount of proteins. As a loading control, the same blots were reprobed with an antibody against α-tubulin. *C,* time course of IL-1-inducible NF-κB DNA binding activity. The experiments were performed as in *B*.

AP-1 were reproducibly lower ($n = 6$) in IκBβ cells compared with other clones. Similar results were obtained with interleukin-11 (IL-11) promoter/CAT reporter with an AP-1-binding site (data not shown). The activity of the reporter gene is AP-1-dependent since an IL-11/CAT reporter in which the AP-1-binding site is mutated was inactive (data not shown). To confirm these results further, we performed transient transfection assays with AP-1/CAT reporter, RelA, and IκB expression vectors in MDA-MB-231 cells. Because of a higher level of basal AP-1 activity, only a modest increase in AP-1/CAT activity in RelA-transfected cells was observed (Fig. 4*C*). Both IκBα and IκBβ inhibited RelA-mediated increases in AP-1/CAT activity.

PEST Domain Mutants of IκBβ Inhibit NF-κB Activity in MDA-MB-231 Cells—Recent studies have indicated that the hypophosphorylated form of IκBβ forms a stable complex with DNA-bound NF-κB (14, 15, 29). Phosphorylation of Ser-313 and Ser-315 of IκBβ by casein kinase II results in the release of both IκBβ and NF-κB from DNA and, consequently, reduced transactivation in P19 and 293 cells (17, 29). Alternatively, promoter context-dependent interaction of HMG I with the IκBβ·NF-κB·DNA complex results in the release of NF-κB and IκBβ from DNA (17). Neither of the above two mechanisms appears to be responsible for reduced NF-κB/CAT activity in IκBβ1-overexpressing MDA-MB-231 cells because transactivation function but not the DNA binding of NF-κB was inhibited by IκBβ1 in these cells. To verify this further, we compared the ability of wild type and mutants of IκBβ (in which casein kinase II phosphorylation sites have been mutated to alanine or aspartic acid) to repress NF-κB DNA binding activity in MDA-MB-231 and 293 cells. The effect of IκBβ1 expression vector lacking 50 amino acids at the C terminus (mIκBβ1ΔC which lacks casein kinase II phosphorylation sites) on NF-κB DNA

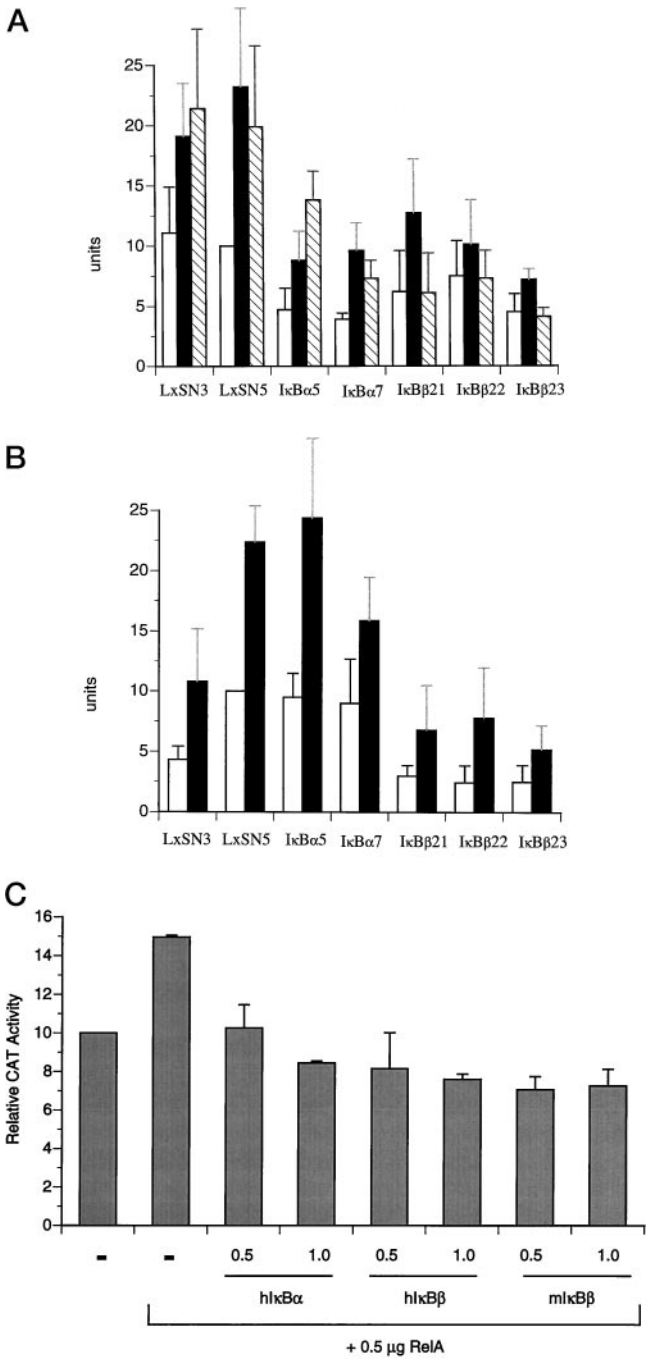

FIG. 4. **Constitutive and inducible activity of NF-κB- and AP-1-dependent promoters in IκB-overexpressing clones.** *A,* activity of NF-κB-dependent promoter. NF-κB/CAT reporter (5 μg) was transfected along with an internal control plasmid coding for β-galactosidase into indicated cells. Cells were treated with either TPA (125 nM) or IL-1β (2.5 ng/ml) 24 h after transfection. CAT activity in equal number of β-galactosidase units was measured 48 h after transfection. Constitutive NF-κB/CAT activity in LxSN5 was set arbitrarily as 10 units, and the relative difference is shown. The mean and standard deviations from four experiments are shown. Basal, TPA- and IL-1-inducible NF-κB activities were significantly lower in IκBα- and IκBβ1-overexpressing cells compared with LxSN cells ($p < 0.0005$). *B,* activity of AP-1-dependent promoter. AP-1/CAT (20 μg) was transfected into indicated clones, and the CAT activity was measured as above. Basal and TPA-inducible AP-1 activities were significantly lower in IκBβ1 cells compared with other cells ($p < 0.0019$). □, untreated; ■, TPA; ▨, IL-1. *C,* RelA increases AP-1/CAT activity. MDA-MB-231 cells were transfected with 10 μg of AP-1/CAT and indicated expression vectors. The amount of expression vector was kept constant in all transfections by substituting with the empty expression vector pcDNA3. Repression of RelA induced NF-κB activity by IκBs was statistically significant ($p < 0.0018$) except in cells transfected with 0.5 μg of IκBα.

binding activity was also tested in parallel. Wild type but not the mutants of IκBβ1 repressed NF-κB DNA binding activity in 293 cells, which is consistent with previously published data (Fig. 5*A*). In contrast, both wild type and mutant IκBβ1 failed to repress NF-κB DNA binding in MDA-MB-231 cells. The C-terminal serines of IκBβ1 appear to be phosphorylated in both cell types as the mutant proteins with either serine to alanine or serine to aspartic acid substitution displayed faster mobility than wild type IκBβ1 on an SDS-polyacrylamide gel electrophoresis (Fig. 5*A*). We next compared the ability of wild type and mutant IκBβ1 to repress transactivation function of NF-κB in transient transfection assays. Mutants were as efficient as wild type IκBβ1 in repressing NF-κB activity (Fig. 5*B*). MDA-MB-231 cells stably expressing mIκBβ1ΔC were generated to confirm further these results (Fig. 5*C*). A new clone containing vector alone was also prepared so that all clones were of the same passage number. mIκBβ1ΔC overexpression did not affect constitutive NF-κB DNA binding activity (Fig. 5*C*). As with the transient transfection assays, however, NF-κB/CAT activity was reduced in clones overexpressing IκBβ1ΔC (Fig. 5*D*). Taken together, these results suggest that phosphorylation of IκBβ1 by casein kinase II is not essential for repression of NF-κB activity in MDA-MB-231 cells.

Serum and EGF-inducible Expression of uPA in IκBα and IκBβ-overexpressing Cells—Multiple transcription factors including AP-1, NF-κB, and the Ets are involved in constitutive and inducible expression of the uPA gene (39, 40). If the effect of IκBβ on NF-κB- and AP-1-dependent gene expression observed in transient assays is relevant to endogenous gene expression, IκBβ overexpression should inhibit uPA gene expression. Serum and EGF were used as inducible agents as both can induce the activity of AP-1 and NF-κB (41, 42). The basal expression level of uPA was consistently lower in LxSN3 compared with LxSN5, possibly due to the difference in passage number (Fig. 6). Both constitutive and inducible expression of uPA was reproducibly lower in IκBα and IκBβ cells (Fig. 6, *A* and *B*). In general, the inducible expression was lower in IκBβ cells compared with IκBα cells. Similar results were also obtained with cells overexpressing IκBβ1ΔC (Fig. 6*C*). The effect of EGF and serum on AP-1 and NF-κB is mostly at the level of transactivation as DNA binding of both transcription factors was not significantly affected upon EGF and serum treatment (data not shown). These results further emphasize that IκBβ1 can repress transcription without interfering with the DNA binding of NF-κB in MDA-MB-231 cells.

uPA Expression in Breast Cancer Cell Lines—The above results indicate that NF-κB plays an important role in uPA expression in breast cancer cells. To extend this observation further, we compared uPA expression with constitutive NF-κB DNA binding activity in various breast cancer cell lines. uPA expression was observed only in cell lines that contained either intermediate or higher levels of constitutive NF-κB activity (Fig. 7). Interestingly, uPA expression in three out of five cell lines correlated with reduced levels of IκBβ (MDA-MB-231, MDA-MB-436, and Hs578T). These results suggest that for optimum activation of NF-κB-regulated genes, constitutive NF-κB DNA binding should be accompanied by reduced levels of IκBβ1.

DISCUSSION

Members of the Rel family of transcription factors are usually sequestered in the cytoplasm by IκB proteins. Several recent studies have indicated that IκB-mediated cytoplasmic retention of NF-κB is altered during cancer progression. Although reduced levels of IκB proteins may be responsible for nuclear NF-κB in some cells, constitutive NF-κB DNA binding has also been observed under conditions where IκBα is abun-

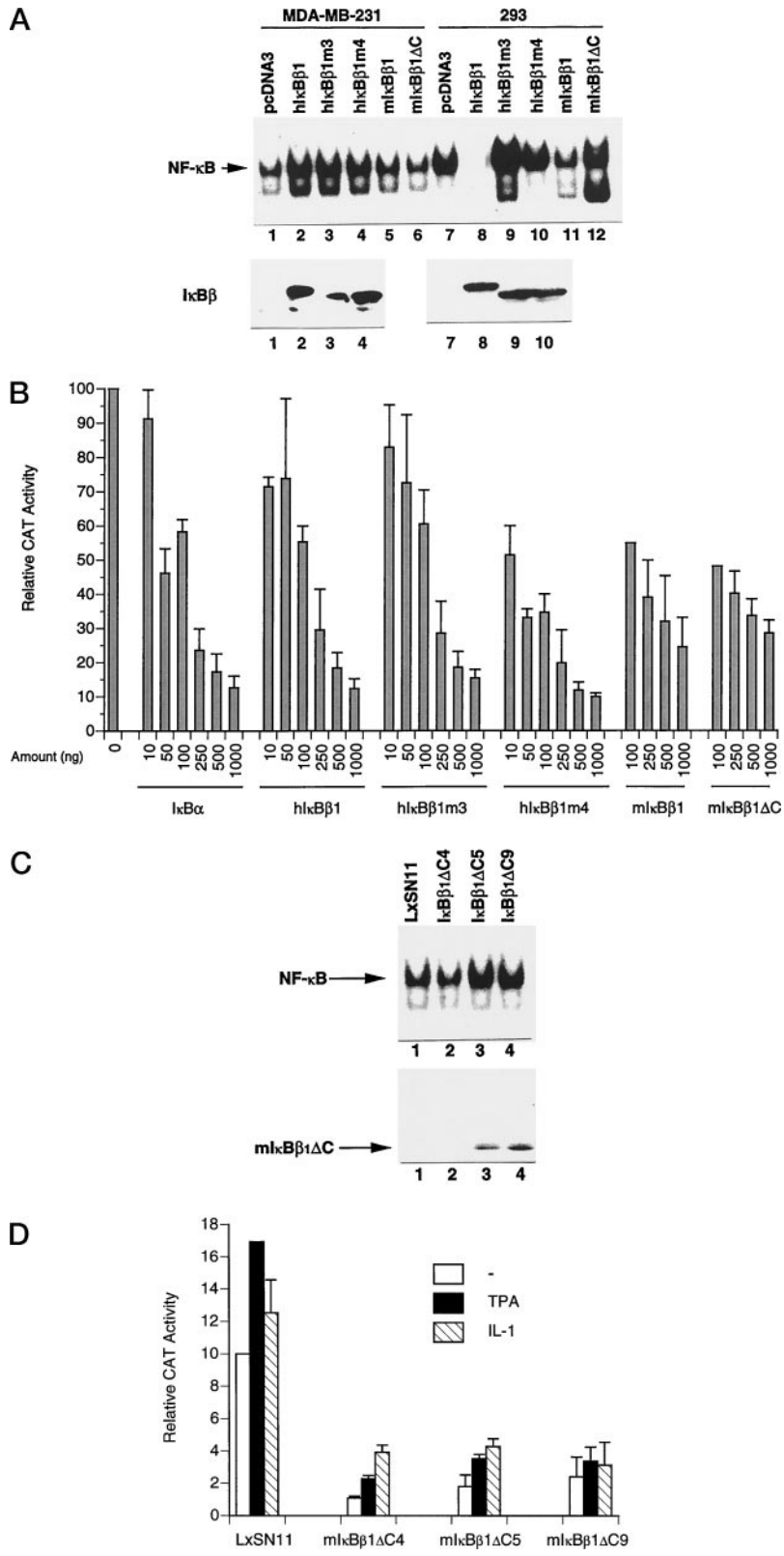

FIG. 5. **Inhibition of NF-κB activity by PEST domain mutants of IκBβ1.** *A,* effect of PEST domain mutants of IκBβ1 on DNA binding of NF-κB in MDA-MB-231 and 293 cells. Cells (5×10^5) were transfected with 10 μg of the indicated expression vectors. Whole cell extracts were prepared after 48 h of transfection and subjected to EMSA (*top panel*) and Western blotting (*bottom panel*). mIκBβ1 and mIκBβ1ΔC could not be detected in Western blots. Note the faster mobility of PEST domain mutants compared with wild type IκBβ1. *B,* PEST domain mutants of IκBβ1 are as efficient as wild type IκBβ1 in repressing NF-κB-dependent promoter activity. MDA-MB-231 cells were transfected with the NF-κB/CAT reporter gene (5 μg) and an indicated expression vector. The amount of expression vector was kept constant in all transfections by substituting with the empty expression vector pcDNA3. CAT activity was measured 48 h after transfection. *C,* generation of stable MDA-MB-231 cells expressing mIκBβ1ΔC. Stable cell lines were generated by retrovirus-mediated gene transfer. The expression level of transduced gene and its effect on NF-κB DNA binding activity were measured by Western blotting and EMSA, respectively. *D*, activity of NF-κB/CAT in IκBβ1ΔC-expressing cells. Transfection and CAT assays were as in Fig. 4.

dant (24, 43). Tyrosine phosphorylation-mediated inactivation without degradation of IκBα or accumulation of hypophosphorylated IκBβ which transports NF-κB to the nucleus may account for constitutive NF-κB DNA binding in cells with IκBα (15, 43). This study was initiated to understand the mechanisms of constitutive NF-κB activation in breast cancer cells. Constitutive NF-κB DNA binding was observed in cells that contained reduced level of IκBα (MDA-MB-435 and MDA-MB-468), IκBβ2 (MDA-MB-436), or both IκBα and IκBβ (MDA-MB-231). Expectedly, overexpression of IκBα could reverse constitutive NF-κB DNA binding in MDA-MB-231 cells. However, it was unexpected that overexpressed IκBβ1 cannot inhibit NF-κB DNA binding in MDA-MB-231 cells even though it could efficiently repress NF-κB DNA binding in 293 cells (Fig. 5*A*, see also Ref. 29). The mechanisms responsible for the cell type-specific function of IκBβ1 is not known. Simeonidis *et al.* (44)

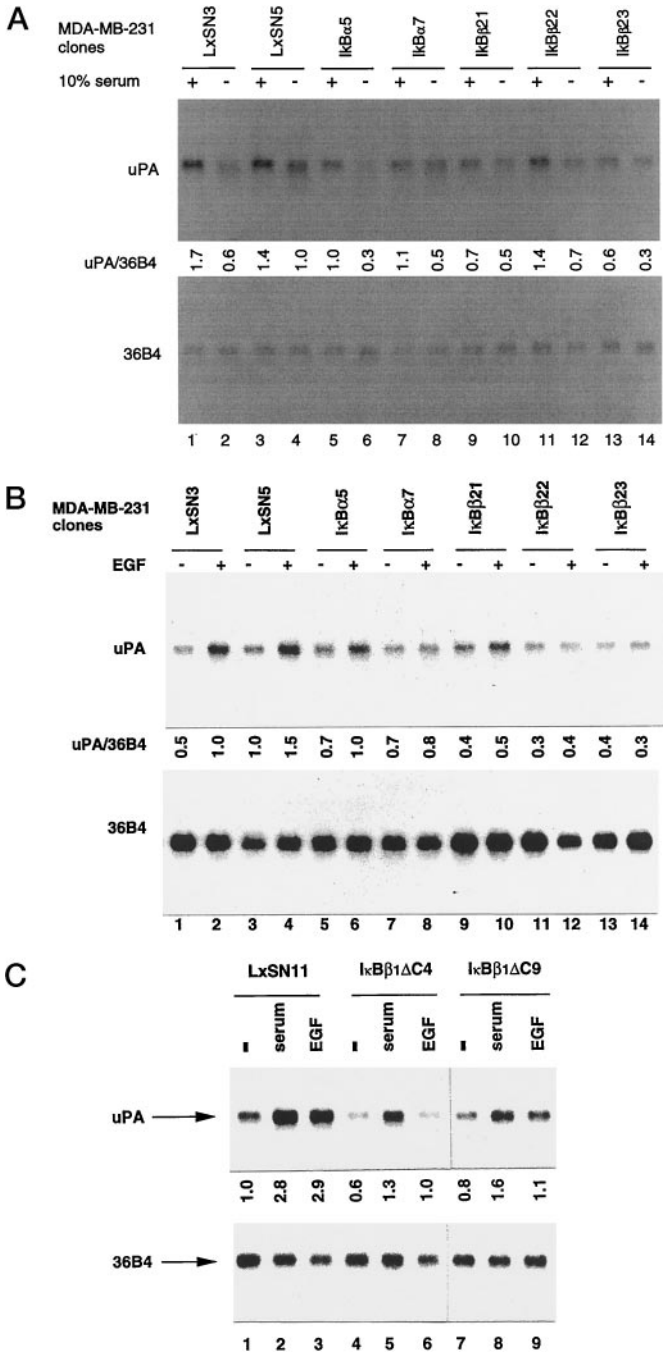

FIG. 6. **Effect of IκBα and IκBβ overexpression on uPA expression.** *A,* serum-inducible expression of uPA. Total RNA (20 μg) from indicated clones maintained in serum-free medium for 48 h (−) or treated for 4 h with 10% serum-containing media after serum starvation (+) was subjected to Northern blotting using a uPA probe. Integrity of the RNA was examined by reprobing the blot with ribosomal protein gene *36B4* (30). The ratio between uPA and 36B4 was determined by densitometric scanning of autoradiograms. Results from a representative experiment are shown. *B,* EGF-inducible expression of uPA. Total RNA from untreated (−) or EGF-treated (30 ng/ml for 4 h) was subjected to Northern blotting as above. *C,* serum and EGF-inducible expression of uPA in cells overexpressing mIκBβ1ΔC.

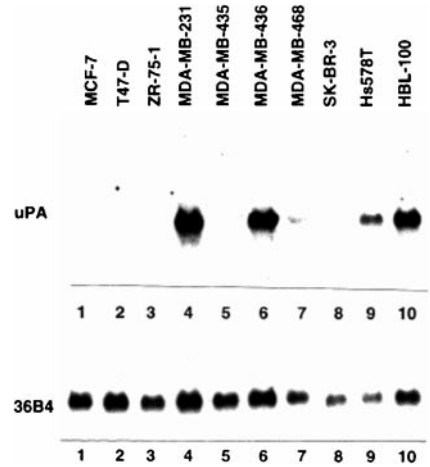

FIG. 7. **Expression of uPA in breast cancer cell lines.** uPA expression was measured by Northern blotting.

have shown that the strength by which different IκBs inhibit NF-κB activity correlates with their ability to sequester NF-κB in the cytoplasm rather than their ability to inhibit DNA binding of NF-κB. They have also shown that the first three of six ankarin repeats determine the cytoplasmic retention potential of IκBs, whereas the last three ankarin repeats and the C-terminal PEST sequences determine the ability of IκBs to inhibit DNA binding. IκBβ1 could neither prevent nuclear translocation nor prevent DNA binding of NF-κB in MDA-MB-231 cells (Fig. 2 and Fig. 5). IκBβ1 was, however, able to inhibit transactivation by NF-κB in these cells. Phosphorylation of PEST domain of IκBβ1 by casein kinase II (which is required for inhibition of DNA binding (16, 17)) is not necessary for repression of transactivation function of NF-κB by IκBβ1 in MDA-MB-231 cells. We observed similar degrees of repression of NF-κB activity by wild type and mutant IκBβ1 lacking casein kinase II phosphorylation sites (Fig. 5). These results suggest that IκBβ1 contains additional functional domains which only inhibit the transactivation function of NF-κB. Detailed mutational analysis and domain swapping with other IκBs is essential for characterization of this domain. We have not directly tested whether this function of IκBβ1 is promoter context-dependent. In this regard, note that IκBβ1 reduced the expression of uPA and the transiently transfected reporter gene but failed to prevent the resynthesis of IκBα (which is dependent on NF-κB) in cells treated with TPA (Fig. 3 and Fig. 6). Because the NF-κB-binding site in the reporter gene and uPA gene lacks the binding sites for HMG I, the repression of NF-κB activity is not likely due to the interaction of HMG I with NF-κB-IκBβ-DNA.

IκBβ may inhibit transactivation by NF-κB independent of its interaction with NF-κB or through its association with DNA-bound NF-κB. IκBβ1 may sequester a coactivator(s) which is essential for transactivation by NF-κB. In this regard, proteins distinct from the IκB/NF-κB family have been shown to interact with IκBβ1 (45, 46). IκBβ1 in the NF-κB·DNA complex may inhibit transactivation in the following ways. (i) IκBβ1 may mask the region of RelA which undergoes a modification such as phosphorylation after DNA binding. Several recent studies have suggested that RelA phosphorylation is required for efficient transactivation. For example, Bergmann *et al.* (47) have demonstrated that inhibitors of phosphatidylcholine-specific phospholipase C and protein kinase C blocked transactivation by NF-κB after TNF and IL-1β treatment even though these inhibitors did not block nuclear translocation. Similarly, Yoza *et al.* (48) have demonstrated that activation of the protein-tyrosine kinase pathway is required for lipopolysaccharide-mediated NF-κB activation but not nuclear translocation. Phosphorylation of RelA by kinases activated by TNF-α and *ras* oncogene has also been reported (49, 50). RelA appears to be the target of protein kinase A or casein kinase II following degradation of IκBs (51, 52). Protein kinase A-mediated phosphorylation by RelA is required for unmasking of intramolecular interaction sites and subsequent interaction of RelA with

coactivator p300/CBP (53). It is possible that phosphorylation by protein kinase A and other kinases and/or coactivator-NF-κB interaction is inefficient when IκBβ1 is present in the NF-κB·DNA complex. (ii) IκBβ has been shown to interact with nuclear receptors including thyroid hormone receptors and retinoid-X receptors (45, 46). Because nuclear receptors such as estrogen receptor, progesterone receptors, and peroxisome proliferator receptors have been shown to inhibit NF-κB activity, IκBβ1 in the NF-κB·DNA complex may recruit retinoid-X receptors:peroxisome proliferator receptor heterodimers to the NF-κB·DNA complex and inhibit transactivation by NF-κB (27, 54–56). (iii) IκBβ1 may inhibit synergistic interaction of NF-κB with other transcription factors including AP-1. We have provided some evidence for the last possibility. Synergistic cross-talk between NF-κB and AP-1 is essential for anchorage-independent growth of immortalized keratinocytes (57). Inhibitors of both NF-κB and AP-1 block tumor promoter-induced transformation (58), suggesting the importance of cross-talk in cancer progression. We suspect that such cross-talk is more pronounced in cancer cells lacking IκBβ1. These cells will be more responsive to growth factors and serum. Consistent with this possibility, serum and EGF-inducible expression of uPA was more pronounced in cells lacking IκBβ1. We have observed increased uPA expression in most of the breast cancer cell lines that contain constitutively active NF-κB. uPA is absolutely required for intravasation, the most important step in the multistep process of tumor metastasis (59), and its overexpression in breast cancer cells is associated with poor prognosis (60). In summation, our results suggest that IκBβ regulates NF-κB activity in a cell type-specific manner which determines whether constitutive DNA binding of NF-κB leads to overexpression of NF-κB-regulated genes such as uPA.

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Negative Regulation of Transactivation Function but Not DNA Binding of NF-κB and AP-1 by I κBβ1 in Breast Cancer Cells

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