

Transforming Growth Factor- β 1-Mediated Activation of NF- κ B Contributes to Enhanced ADAM-12 Expression in Mammary Carcinoma Cells

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Abstract

A disintegrin and metalloproteinase-12 (ADAM-12), a member of multifunctional family of proteins, is upregulated in many cancers, including breast, lung, liver, prostate, gastric, and bladder. The multidomain structure, composed of a prodomain, a metalloproteinase, disintegrin-like, epidermal growth factor-like, cysteine-rich and transmembrane domains, and a cytoplasmic tail, allows ADAM-12 to promote matrix degradation, cell-cell adhesion, and intracellular signaling capacities and thereby to play a critical role in cancer growth and metastasis. Despite ample evidence linking increased ADAM-12 expression with cancer, the mechanisms controlling its upregulation are still unknown. In the present study, transforming growth factor- β 1 (TGF- β 1) is shown to increase ADAM-12 mRNA expression in MDA-MB-231 breast carcinoma cells. We have identified a promoter element responsible for TGF- β 1-mediated *ADAM-12* induction. We show interaction of NF- κ B with *ADAM-12* promoter and that high level of NF- κ B activity in breast carcinoma cells results in the upregulation of *ADAM-12* expression. Site-directed mutagenesis of the NF- κ B element in *ADAM-12* promoter and inhibition of NF- κ B activity by Bay-11-7085 and MG-132 significantly reduced TGF- β 1-mediated increase of *ADAM-12* promoter-driven gene expression. Transfection of cells with a dominant-negative mutant form of I κ B α (I κ B α Δ N), which inhibits activation of NF- κ B, significantly reduced transcription from *ADAM-12* promoter-reporter in TGF- β 1-stimulated MDA-MB-231 cancer cells. In correlation, overexpression of NF- κ B induced *ADAM-12* expression in a dose-dependent manner. DNA-binding and ChIP assays indicated that p65 subunit of NF- κ B binds to *ADAM-12* promoter. Together, our study identified a cellular mechanism for induction of *ADAM-12*, which involves NF- κ B and its activation by TGF- β 1. *Mol Cancer Res*; 8(9); 1261–70. ©2010 AACR.

Introduction

ADAM-12, also known as meltrin- α , is a key member of the large (>33 members) ADAM (a disintegrin and metalloproteinase) family of proteins, which are capable of doing several biological functions, including proteolysis, regulation of growth factor availability, cell-cell and cell-matrix adhesion, cell signaling, and ectodomain shedding (1). ADAM-12 protein contains multiple structural domains consisting of (from the NH₂-terminal to the COOH-terminal) a prodomain, metalloproteinase domain, disintegrin domain, cysteine-rich domain, epidermal growth factor (EGF)-like transmembrane domain, and cytoplas-

mic domain. Multidomain structure allows ADAM-12 to do various physiologic tasks. ADAM-12 is shown to be involved in cell-cell and cell-matrix interactions, leading to myogenesis (2) and adipogenesis (3), and to promote EGF receptor signaling by shedding of the heparin-binding EGF (HB-EGF) from the membrane-anchored inactive precursor pro-HG-EGF form (4) and release of insulin-like growth factor binding proteins 3 and 5 (IGFBP-3 and IGFBP-5; refs. 5, 6). Although biological significance of ADAM-12 is yet to be fully understood, an increase in the level of ADAM-12 is detected during several pathologies (7). In many tumor tissues, including breast cancer (8), liver cancer (9), gastric cancer (10), brain cancer (glioblastoma; ref. 11), bone cancer (12), and prostate cancer (13), ADAM-12 is seen to be upregulated. Furthermore, ADAM-12 is shown to support tumor cell adhesion (14). Increase of urinary ADAM-12 protein level correlating with breast cancer progression (15), lower rate of apoptosis (16), and increase of cell proliferation in cancer cells during overexpression of ADAM-12 (17) all implicate for a potential role of ADAM-12 in cancer. In addition to cancer, upregulation of ADAM-12 is linked to osteoarthritis (18, 19), chronic wound-healing process (20), and cardiac hypertrophy (4).

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In most adult tissues, with the exception of some developmental and physiologic conditions, ADAM-12 expression is very low and tightly regulated. For example, neonatal skeletal muscle expresses ADAM-12 at a very high level but adult skeletal muscle expresses ADAM-12 at a low basal level (21). During pregnancy, ADAM-12 level is induced in placental tissue and maternal serum (2). In human, two alternatively spliced forms of ADAM-12 have been identified, in which the longer form (ADAM-12L) remains associated with membrane, whereas the shorter form (ADAM-12S) is secreted (2). Whereas ADAM-12 structure and function have been extensively investigated, its transcriptional regulation under physiologic or pathologic conditions remains practically unknown.

To understand the molecular mechanisms controlling increase of *ADAM-12* gene expression in cancer, we have characterized human *ADAM-12* promoter and its activation. With combined use of transient transfection, site-directed mutagenesis, and electromobility shift and chromatin immunoprecipitation assays, we show that NF- κ B, which is activated in breast cancer cells following transforming growth factor- β 1 (TGF- β 1) stimulation, is involved in mediating enhanced expression of *ADAM-12* gene. These studies could help in the development of new therapeutic strategies associated with abnormal ADAM-12 expression in cancer.

Materials and Methods

Cell culture

Human MDA-MB-231 breast cancer cells and MCF-10A normal mammary epithelial cells were obtained from the American Type Culture Collection and maintained in DMEM containing high glucose, 100 units/mL penicillin, and 100 units/mL streptomycin supplemented with 10% FCS.

Isolation of RNA and Northern blot analysis

Total RNA was isolated by using guanidinium thiocyanate method (22). MDA-MB-231 cells were either left untreated or treated with 5 or 20 ng/mL of TGF- β 1 (Sigma Chemical Co.) for up to 24 hours. Additionally, some cells were treated with anti-TGF- β 1 antibody (20 μ g/mL; R&D Systems) or a nonspecific antibody (20 μ g/mL). In some assays, MDA-MB-231 cells were transfected with I κ B α Δ N, which is a dominant-negative mutant form of I κ B α . Total RNA (50 μ g) was fractionated in 1.0% agarose gel containing 2.2 mol/L formaldehyde, transferred onto a nylon membrane, and hybridized with a PCR-amplified ADAM-12 cDNA probe (2). To ensure the quality and quantity of each RNA sample, the same blot was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. For detection of radiolabeled hybridized probes, the membranes were exposed to X-ray films and autoradiographed. For estimation of relative level of ADAM-12 mRNA in different RNA preparations, the autoradiographs were scanned by a densitometer and values were plotted as ratio of ADAM-12 to GAPDH.

Plasmid constructs

Approximately, a 1.95-kb-long DNA fragment containing the 5'-flanking region of human *ADAM-12* was cloned in pTZ19U vector by PCR amplification of human genomic DNA. The primers used for PCR were 5'-CCAC-CAGGTCCCTCCCACAACACATG-3' (forward) and 5'-TAGTTCGGCGACTTAGGTTCGGGC-3' (reverse). The human *ADAM-12* promoter DNA was sequenced and compared with the human genomic sequence for verification (Genbank/European Molecular Biology Laboratory database accession no. AL589787). The 1.6ADAM-12CAT3 reporter gene was constructed by cloning sequences from -1,600 to +19 of human *ADAM-12* promoter into pBLCAT3 vector (23). Progressively deleted ADAM-12 reporter constructs were constructed by cloning various segments of *ADAM-12* promoter that were generated by PCR amplification using different upstream primers starting at nucleotide positions -1,000, -600, -75, and -30 and a single downstream primer ending at position +19 of human *ADAM-12*. Mutant NF- κ B site *ADAM-12* promoter was prepared by megaprimer PCR, in which the sequence of the mutant NF- κ B oligonucleotide was 5'-GACCCATCCCAG-3'. Underlined bases represent altered sequences.

NF- κ B expression plasmids pCMVp65 and pCMVp50 were prepared by inserting a full-length cDNA of each transcription factor in pcDNA3.1 vector (Invitrogen Life Technologies). The FLAG-I κ B α Δ N, a dominant-negative mutant form of I κ B α , in which NH₂-terminal 1 to 36 amino acids are deleted, is cloned in expression plasmid pCMV4 (a generous gift from Dean Ballard, Vanderbilt University, Nashville, TN; ref. 24).

Transfection analysis

MDA-MB-231 cells were transfected by adding reporter plasmid DNA together with pSV β -gal (Promega) plasmid DNA by Lipofectamine method as described (25). In some cotransfection experiments, various amounts of expression plasmid DNAs for pCMVp65, pCMVp50, and/or pCMVI κ B α Δ N were added together with the reporter plasmid DNA. The amount of DNA in each transfection assay was kept the same by adding carrier plasmid DNA. The pSV β -gal DNA was used to monitor the efficiency of transfection and to normalize the cell extracts used for CAT assay. The cells were stimulated with TGF- β 1 (20 ng/mL) and harvested 24 hours later. In some transfection assays, MG-132 (20 μ mol/L) or Bay-11-7085 (20 μ mol/L) was added. CAT and β -galactosidase activities were measured as described (25). Before the CAT assay, each cell extract was heated at 60°C for 10 minutes to inactivate endogenous acetylase activity. Different agents used in the transfection assay had no effect on β -galactosidase expression. All transfection experiments were done at least three times.

Nuclear extract preparation and electromobility shift assay

MDA-MB-231 and MCF-10A cells were grown in 100-mm dishes in the presence or absence of TGF- β 1,

which was added at different concentrations as described in the figure legends and incubated for 24 hours. The cells were harvested by scrapping, and nuclear extracts were prepared as described earlier (26). The oligonucleotides used as probes contained sequences from positions -336 to -314 of *ADAM-12* promoter. The sequences of the wild-type and mutant oligonucleotides were 5'-GCACTCCGAGGGTTCCAGAGAG-3' and 5'-GCACTCCGACCCATCCCAGAGAG-3', respectively. Underlined bases in the mutant oligonucleotide represent altered bases. For authentic consensus NF- κ B probe, immunoglobulin κ B enhancer oligonucleotide containing sequences 5'-GATCCAGAGGGGACTTCCGAGA-3', described earlier (27), was used. All oligonucleotide probes for mobility shift assay were double-stranded and labeled by Klenow fill-in reaction with [α - 32 P]dCTP, and binding reactions were done using 10 μ g protein of nuclear extracts following a method described earlier (26). Some binding reactions contained molar excess of nonradioactive oligonucleotides with wild-type or mutant NF- κ B-binding elements. For antibody interaction studies, anti-p65 NF- κ B or anti-p50 NF- κ B, both rabbit polyclonal IgG, or normal rabbit IgG (Santa Cruz Biotechnology) was added to the binding reaction mixture during a preincubation for 30 minutes on ice. Anti-p65 antibody was developed against an epitope representing the NH₂ terminus of NF- κ B p65 (RelA) of human origin, and anti-p50 antibody was developed against the nuclear localization signal region of NF- κ B p50 (NF κ B1) of human origin.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was done following a method described earlier (28) with a ChIP assay kit (Upstate Biotechnology) following the manufacturer's protocol. Briefly, MDA-MB-231 cells (2×10^6 each) were left either untreated or stimulated with TGF- β 1 (20 ng/mL). Twenty-four hours later, formaldehyde (1% final concentration) was directly added to the culture medium for cross-link and incubated at 37°C for 10 minutes. Cells were washed with ice-cold PBS, lysed in 200 μ L of cell lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 1% SDS] with a protease inhibitor mixture, and sonicated to generate on average 500-bp-long DNA fragments, and the supernatants were diluted with dilution buffer [20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitors]. The solutions were precleared with salmon sperm DNA/protein G agarose slurry and then incubated with anti-p65 NF- κ B antibody, anti-p50 NF- κ B antibody, or control IgG (Santa Cruz Biotechnology) at 4°C for 16 hours with rotation. The beads were washed sequentially in low-salt, high-salt, LiCl, and Tris-EDTA buffer. Immune complexes were extracted from the beads with 1% SDS and 0.1 mol/L NaHCO₃. Cross-linking was reversed by heating at 65°C for 4 hours. The eluates were then digested with proteinase K and RNase A, and the DNA was phenol extracted and ethanol precipitated. Purified DNA was subjected to PCR using primers cov-

ering the NF- κ B-binding region of *ADAM-12* promoter. PCR products were resolved in a 2% agarose gel and visualized by ethidium bromide staining. The primers used for amplification of NF- κ B-binding element of *ADAM-12* gene were 5'-TTTGGGCGCTTAGGGCCGGGC-3' (sense) and 5'-ATGCGGCGGCACGGTCCAGC-3' (antisense), which yields a 152-bp product.

Western blotting

Cell extracts (50 μ g) were fractionated in SDS-11% PAGE and electroblotted onto a nitrocellulose membrane. Immunoblotting was done using anti-FLAG antibody (Sigma-Aldrich Biotechnology) as the primary antibody for I κ B α Δ N protein detection and horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody. In some assays, anti-*ADAM-12* and anti- β -actin antibodies, both were rabbit polyclonal IgG and developed against human proteins (Santa Cruz Biotechnology), were used as primary antibodies. Bands were detected using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Results

Induction of *ADAM-12* by TGF- β 1

Most mammary tumors have been shown to contain high level of TGF- β . Further, most (>78%) of the estrogen receptor-negative (ER⁻) tumors exhibit high level of TGF- β , which is critical for increased proteolytic activity and metastatic potential of mammary tumor cells (29, 30). MDA-MB-231 cells, an ER⁻ mammary tumor cells, express TGF- β 1 and TGF- β 2 (31). Northern blot analysis indicated that TGF- β 1 stimulates expression of *ADAM-12* mRNA in MDA-MB-231 cells in a dose-dependent manner (Fig. 1A, lanes 2 and 3, and D). In correlation, reduction of endogenous TGF- β 1 level by the addition of a monoclonal TGF- β 1 antibody in the culture medium significantly lowered *ADAM-12* mRNA level compared with nonspecific IgG-treated or untreated MDA-MB-231 cells (Fig. 1B, compare lanes 1-3, and E). A time course analysis of induction revealed a significant increase of *ADAM-12* expression at 16-hour time point (Fig. 1C and F). *ADAM-12* protein level was also increased in response to TGF- β 1 stimulation (Fig. 1G). Together, these data suggested that TGF- β 1 acts as an inducer of *ADAM-12* expression in MDA-MB-231 cancer cells.

Identification of TGF- β 1-responsive regulatory element

To analyze TGF- β 1-mediated transcriptional regulation of *ADAM-12*, we generated a series of progressively deleted *ADAM-12* promoter-CAT constructs (Fig. 2A), which were transiently transfected in MDA-MB-231 cells and incubated with or without TGF- β 1. 1.6*ADAM-12*CAT, 1.0*ADAM-12*CAT, and 0.6*ADAM-12*CAT exhibited quite similar type of responsiveness (~4.5-fold induction) to TGF- β 1, whereas 0.07*ADAM-12*CAT exhibited almost no TGF- β 1 responsiveness (Fig. 2B). These results indicated the possible presence of TGF- β 1-responsive element(s) within -600- and -75-nucleotide position in the human

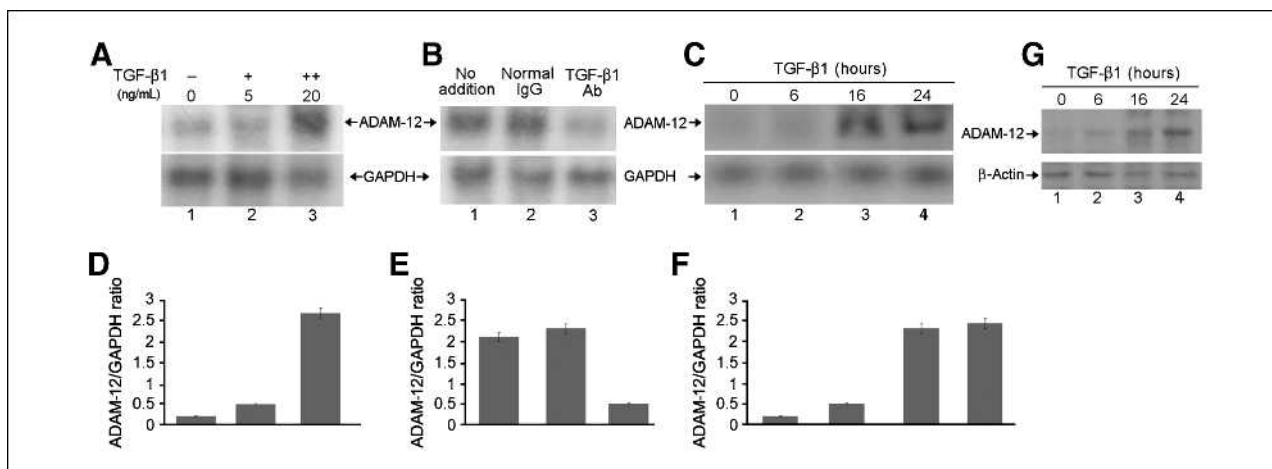


FIGURE 1. Upregulation of *ADAM-12* by TGF- β 1. **A**, effect of TGF- β 1 addition. MDA-MB-231 breast carcinoma cells were left untreated (lane 1) and stimulated with 5 ng/mL TGF- β 1 (lane 2) or 20 ng/mL TGF- β 1 (lane 3), as indicated, for 24 h. Total RNA (50 μ g) isolated from these cells was subjected to Northern blot analysis by using a radiolabeled *ADAM-12* cDNA probe. After removal of the previous radioactive signal, the same blot was rehybridized using a radiolabeled GAPDH cDNA probe, which was used as normalization or loading control. **B**, neutralizing effect of anti-TGF- β 1 antibody (Ab). MDA-MB-231 cells were left untreated (lane 1) and treated with a normal rabbit IgG (20 μ g/mL) or an anti-TGF- β 1 antibody (20 μ g/mL) for 24 h. The same amount of RNA was used in Northern blot analysis and probed similarly as described in **A**. **C**, MDA-MB-231 cells were either left untreated (lane 1) or treated with 20 ng/mL TGF- β 1 for 6, 16, and 24 h (lanes 2-4). Samples (50 μ g) of total RNA isolated from these cells were used in Northern blot analysis and probed as described in **A**. **D** to **F**, densitometric analysis of autoradiographs of the samples probed with *ADAM-12* and GAPDH as shown in **A** to **C**, respectively. Columns, mean from four independent measurements; bars, SD. **G**, effect of TGF- β 1 addition on *ADAM-12* protein expression. Total protein (50 μ g), isolated from MDA-MB-231 cells treated without (lane 1) or with 20 ng/mL of TGF- β 1 for 6, 16, and 24 h (lanes 2-4), was subjected to Western blot analysis by using anti-*ADAM-12* antibody. Blots were developed with enhanced chemiluminescence reagent. The membrane was stripped and reprobed with β -actin antibody to confirm equal loading.

ADAM-12 promoter. Further deletion of upstream sequences up to nucleotide position -30 resulted in severe drop in basal expression of the reporter gene.

Dominant-negative I κ B α blocks TGF- β 1-mediated induction of *ADAM-12*

To probe further into the nature of the regulatory elements present in the TGF- β 1-responsive region of the

ADAM-12 promoter, the sequence of this region (-600/-75) was examined, which revealed a putative NF- κ B-binding sequence (5'-GAGGGTTCAG-3') present between -329 and -318. To examine whether TGF- β 1-induced *ADAM-12* expression occurs through the NF- κ B/I κ B α pathways, we suppressed activation of NF- κ B by transient transfection of the cells with a dominant-negative mutant form of I κ B α (pCMV4-FLAG-I κ B α Δ N)

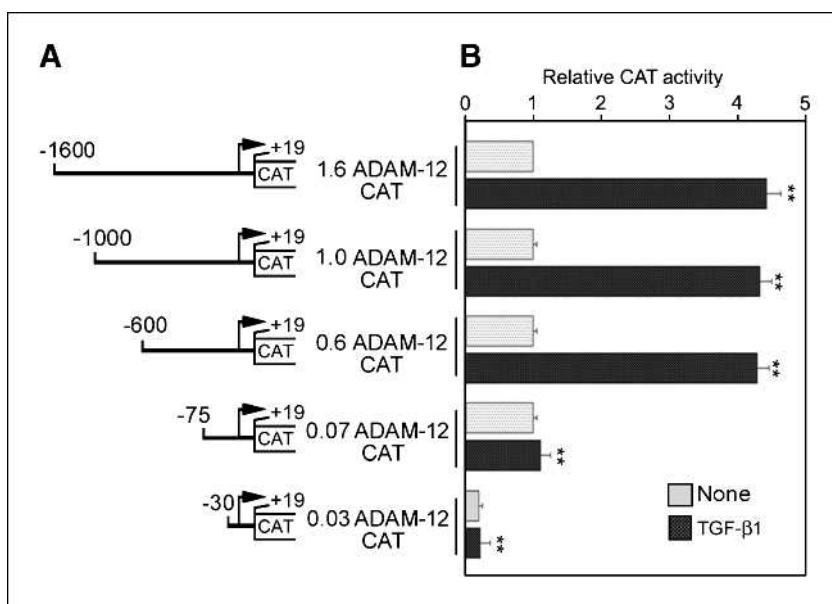


FIGURE 2. Human *ADAM-12* promoter is activated by TGF- β 1 and involves an NF- κ B element. **A**, schematic representation of progressively deleted *ADAM-12* promoter fragments cloned into pBLCAT3 vector. **B**, mapping of the TGF- β 1-responsive regions. MDA-MB-231 cells were transiently transfected, in duplicate, with various progressively deleted *ADAM-12*CAT reporter plasmids (0.5 μ g each), as indicated. Following transfection, one set of cells was incubated in the presence of TGF- β 1 (20 ng/mL) and the other set was left untreated for an additional 24 h. Induction of CAT activity in equivalent amount of transfected cells relative to that of uninduced cells was determined and plotted as relative CAT activity. These results represent an average of three independent experiments. **, $P < 0.01$, compared with untreated cells.

expression plasmid (24). The I κ B α Δ N protein remains bound to NF- κ B because of deletion of the NH₂-terminal 1 to 36 amino acids that contains two critical serine residues at positions 32 and 36 required for phosphorylation and proteolytic degradation (24). As expression of I κ B α Δ N repressed TGF- β 1-mediated induction of the 0.6ADAM-12 promoter-reporter construct in a dose-dependent manner, it suggested possible involvement of the NF- κ B signaling pathway (Fig. 3A). Western blot assay with anti-FLAG antibody indicated dose-dependent increased level of I κ B α Δ N protein expression in the transfected cells (Fig. 3B). To evaluate whether TGF- β 1 stimulation activated NF- κ B, DNA-binding activity was measured using an authentic NF- κ B-binding DNA that was originally detected in the immunoglobulin κ B enhancer region (27). As shown in Fig. 3C, nuclear factors in TGF- β 1-stimulated MDA-MB-231 cells interacted with authentic NF- κ B element and the DNA-protein complex was supershifted by anti-p65 NF- κ B antibody (Fig. 3C, lane 4). RNA blot analysis indicated that inhibition of activation of NF- κ B during overexpression of I κ B α Δ N in transfected MDA-MB-231 cells reduces ADAM-12 mRNA level in a dose-dependent manner (Fig. 3D).

NF- κ B element is critical for TGF- β 1-mediated induction of ADAM-12 promoter

For evaluation of the regulatory role of NF- κ B, we introduced selective mutation at the NF- κ B element in the large ADAM-12 promoter. As compared with the wild-type reporter, the mutant reporter (0.6 mut NF- κ B ADAM-12CAT) showed markedly lower response to TGF- β 1 stimulation in transfected MDA-MB-231 cells (Fig. 4). In correlation, addition of Bay-11-7085, which is an inhibitor of I κ B α phosphorylation and thus inhibits activation of NF- κ B, antagonized TGF- β 1-mediated stimulation of the wild-type ADAM-12 reporter. MG-132, which being a proteasome inhibitor prevents proteolytic degradation of I κ B proteins and inhibits activation of NF- κ B, showed similar effect. As a positive control, we transfected MDA-MB-231 cells with 2 \times NF- κ B-CAT2 reporter, where CAT expression is regulated by two tandem bona fide NF- κ B promoter elements. Similar to 0.6ADAM-12CAT, the CAT expression in the positive control plasmid was also induced by TGF- β 1 and inhibited by the inhibitors of NF- κ B. Together, these results confirmed the involvement of NF- κ B in TGF- β 1-mediated regulation of ADAM-12 promoter function.

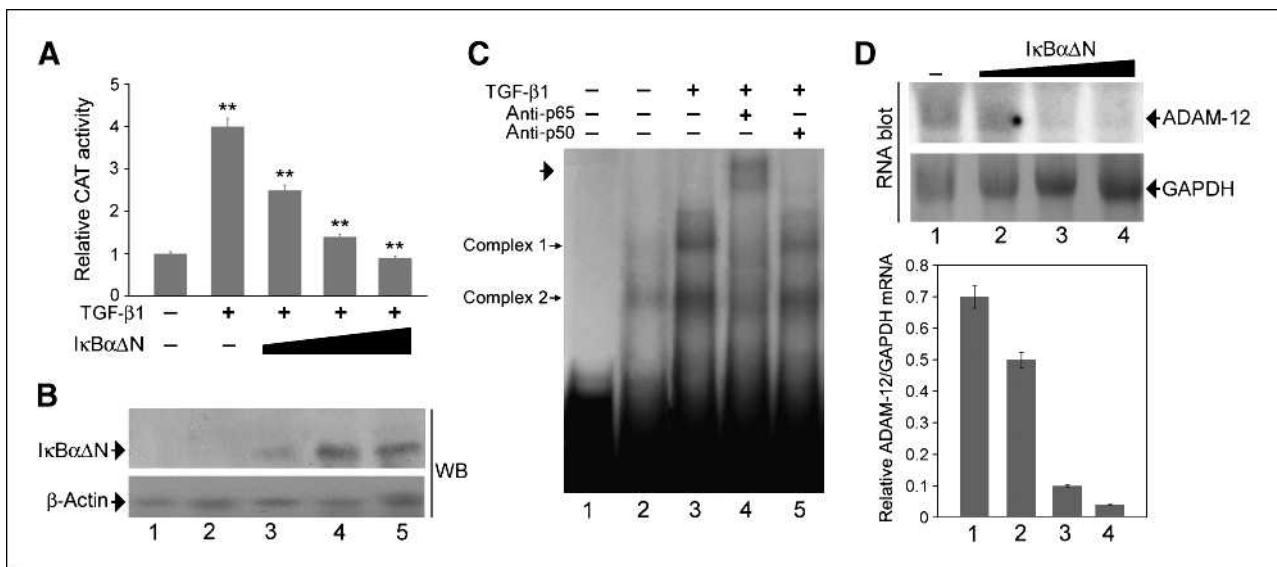


FIGURE 3. Inhibition of NF- κ B activation during overexpression of phosphorylation-defective I κ B α mutant lowers TGF- β 1-mediated stimulation of the human ADAM-12 promoter-reporter. A, MDA-MB-231 cells were transiently transfected, in duplicate, with the 0.6ADAM-12CAT reporter plasmid (0.5 μ g) either alone or with increasing concentrations (0.1, 0.5, and 1.0 μ g) of a FLAG-tagged I κ B α Δ N expression plasmid and further incubated with or without TGF- β 1 (20 ng/mL), as indicated, for additional 24 h. CAT activity in equivalent amount of transfected cells relative to that of uninduced cells was determined and plotted as relative CAT activity. These results represent an average of three independent experiments. **, $P < 0.01$, compared with untreated cells. B, Western blot (WB) assay of transfected cells for detection of I κ B α Δ N protein expression in the same samples as in A. Equal protein amount of cell extracts was immunoblotted with an anti-FLAG antibody. Blots were developed with enhanced chemiluminescence reagent. The membrane was stripped and reprobed with β -actin antibody to confirm equal loading. C, activation of NF- κ B by TGF- β 1 stimulation of MDA-MB-231 cells. Nuclear extracts (10 μ g protein), prepared from MDA-MB-231 cells treated without (lane 2) or with TGF- β 1 (20 ng/mL) for 24 h (lanes 3-5), were incubated with a double-stranded ³²P-labeled authentic NF- κ B DNA probe as described in Materials and Methods. In addition, lanes 4 and 5 contain anti-p65- and anti-p50-specific antibodies (both obtained from Santa Cruz Biotechnology), respectively. Lane 1 contains probe only. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel. Two DNA-protein complexes are designated as complex 1 and 2, respectively. The arrow indicates the supershifted complex in lane 4. D, ADAM-12 mRNA expression in MDA-MB-231 cells in response to I κ B α Δ N protein expression. Total RNA was prepared from cells that were transfected and treated with TGF- β 1, the same way as in B. Total RNA (50 μ g) was used in Northern blot analysis and probed with a radioactive ADAM-12 cDNA and GAPDH cDNA, as described in Fig. 1A. mRNA level of ADAM-12 relative to that of GAPDH was evaluated by densitometric analysis of autoradiographs of the samples probed with ADAM-12 and GAPDH. Columns, mean from four independent measurements; bars, SD.

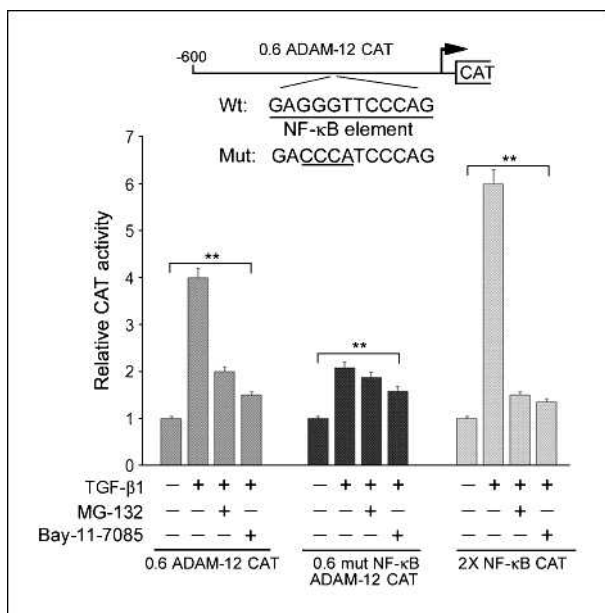


FIGURE 4. Mutation of the NF- κ B element or inhibition of NF- κ B activation is critical for TGF- β 1-mediated upregulation of human *ADAM-12* promoter. The schematic illustrates the wild-type (Wt) *ADAM-12* and mutant (Mut) NF- κ B *ADAM-12* reporter plasmids. MDA-MB-231 cells were transfected with reporter plasmids (0.5 μ g each) and further incubated with TGF- β 1 (20 ng/mL) for an additional 24 h, as indicated. In addition to TGF- β , some transfected cells received MG-132 (20 μ mol/L) and Bay-11-7085 (20 μ mol/L), as indicated. Induction of CAT activity in equivalent amount of transfected cells relative to that of uninduced cells was determined and plotted as relative CAT activity. These results represent an average of three independent experiments. **, $P < 0.01$, compared with untreated cells.

Increased binding of NF- κ B to the *ADAM-12* promoter

Because TGF- β 1 increased *ADAM-12* promoter activity, it begged the question whether altered level of NF- κ B transcription factor binding to the *ADAM-12* NF- κ B element is responsible for this phenomenon. To test this possibility, nuclear extracts of uninduced and TGF- β 1-induced MDA-MB-231 cells were analyzed for DNA-binding activity. Two DNA-protein complexes, I and II, were formed with uninduced cell nuclear extract (Fig. 5A, lane 2), but the levels of both complexes were much higher in TGF- β 1-induced cells, which increased in a dose-dependent manner (Fig. 5A, lanes 3-6). No DNA-binding activity was seen when mutant NF- κ B DNA-binding element was used as a probe (Fig. 5A, lanes 7-11). The DNA-protein complexes were further characterized by competition analysis (Fig. 5B). The DNA-protein complexes I and II were inhibited when unlabeled consensus NF- κ B oligonucleotide was included (Fig. 5B, lanes 2 and 3) and remained unaffected when unlabeled nonspecific oligonucleotide was added (Fig. 5B, lanes 4 and 5). In addition, both complexes were partly inhibited by anti-p50 NF- κ B antibody (lane 6) but supershifted by anti-p65 NF- κ B antibody (lane 7). MDA-MB-231 cells, when transfected with dominant-negative I κ B Δ N expression

plasmid, suppressed TGF- β 1-mediated increase of both DNA-protein complexes (Fig. 5C). Together, the DNA-binding assay suggested that these subunits of NF- κ B and p65 in particular are activated in TGF- β 1-induced MDA-MB-231 cells and bind to the *ADAM-12* promoter at the NF- κ B DNA-binding site located between nucleotide positions -329 and -318.

In vivo interaction of NF- κ B with *ADAM-12* promoter

To examine *in vivo* interaction of NF- κ B proteins with the *ADAM-12* promoter during high-level expression of *ADAM-12* in the mammary carcinoma cells, we analyzed the chromatin organization of the *ADAM-12* promoter in the cells. ChIP analysis indicated that the κ B element of human *ADAM-12* promoter was occupied by p65 NF- κ B transcription factor, selectively in the TGF- β -stimulated cells (Fig. 6A, compare lanes 3 and 6). The specificity of the ChIP assay was verified using control IgG-precipitated chromatin, where no PCR-amplified product was visible (Fig. 6A, lanes 2 and 5). A similar experiment with anti-p50 NF- κ B antibody showed not much visible PCR-amplified product in p50 antibody-precipitated cells during unstimulated or TGF- β 1-stimulated conditions (Fig. 6B, lanes 3 and 6). For further analysis of the role of NF- κ B subunits, we analyzed the effect of p65 NF- κ B and p50 NF- κ B on *ADAM-12* reporter gene. As seen in Fig. 6C, overexpression of p50 NF- κ B in transfected MDA-MB-231 cells had almost no effect, whereas p65 NF- κ B transactivated the same reporter in a dose-dependent manner. Interestingly, although p50 alone showed no transactivating function, it synergized transactivating potential of p65 NF- κ B by producing a robust transcription from *ADAM-12* promoter that was much higher than a simple additive level. In accord, NF- κ B DNA binding with the *ADAM-12* promoter was significantly less in normal mammary epithelial cells, MCF-10A, compared with metastatic MDA-MB-231 breast cancer cells (Fig. 6D).

Discussion

Accumulating evidence indicates a prominent link between the upregulation of *ADAM-12* and cancer. At present, regulatory mechanisms controlling *ADAM-12* transcription, by which *ADAM-12* protein level is increased, are completely unknown. Our studies show that NF- κ B plays a critical role in the upregulation of human *ADAM-12* in MDA-MB-231 breast cancer cells, and present explanation as to how this multifunctional protein is induced in cancer cells. We provide evidence for (a) TGF- β 1-mediated increase of *ADAM-12* expression in MDA-MB-231 cells, (b) involvement of NF- κ B/I κ B pathways, (c) increased interaction of NF- κ B with human *ADAM-12* gene promoter both *in vitro* and *in vivo*, and (d) activation of the human *ADAM-12* promoter by NF- κ B in a subunit-specific manner, and show that (e) specific mutation of the NF- κ B element or (f) inhibition of NF- κ B activation significantly reduces TGF- β 1 responsiveness of human *ADAM-12* promoter.

In the tumor microenvironment, ADAM-12 expression is detected in both tumor and surrounding stromal cells and urinary level of ADAM-12 is seen to correlate with breast cancer status and stage (15). High level of ADAM-12 expression in cancer tissues is believed to facilitate release of membrane-anchored form of HB-EGF that plays a key role in human breast carcinoma cell growth. It is worthy of mention that the EGF family members along with HB-EGF are implicated in tumor cell proliferation and metastasis (32). HB-EGF, which is synthesized as a membrane-anchored form, requires proteolytic processing to become a bioactive molecule, and ADAM-12 is shown to be involved in proteolytic processing of pro-HB-EGF, referred to as shedding (4). In addition, the cell adhesion activity of ADAM-12 due to its interaction with $\alpha_5\beta_1$ integrin and syndecans may also facilitate tumor progression (33). Together, these findings underscore the critical role of ADAM-12 in cancer cell growth.

How ADAM-12 level is increased in cancer cells is the relevant next question. We show that TGF- β 1 may be involved in this process. TGF- β is one of the most widely distributed cytokines/growth factors that act on virtually all cell types and their function (34). The role of TGF- β in controlling cell proliferation, differentiation, migration, apoptosis, and extracellular matrix development is well established (35, 36). In addition, TGF- β is implicated in reg-

ulating inflammatory responses, as knockout of *TGF- β* gene is shown to cause multifocal inflammatory responses resembling autoimmune disease and death (37). However, because of the two different types of effects of TGF- β as reported during cancer, the role of TGF- β in breast cancer still remains elusive. At early stages of the breast cancer, TGF- β is shown to inhibit epithelial cell proliferation and act as a tumor suppressor, whereas at later stages of the disease, TGF- β is shown to function as a tumor promoter (38, 39). Furthermore, the action of TGF- β is shown to be dependent on the ER status of the breast cancer cells. In ER⁺ MCF-7 cells, TGF- β inhibits cancer cell proliferation (40), whereas in ER⁻ MDA-MB-231 cells, TGF- β stimulates cancer cell proliferation (41).

Our finding of the activation of NF- κ B in MDA-MB-231 cells in response to TGF- β 1 treatment raises an interesting possibility whereby growth factor-mediated signal acts in cooperation with NF- κ B in the metastatic cancer cells, whereas such cooperation is absent in MCF-10A normal breast epithelial cells. It is possible that in the metastatic cancer cells, TGF- β 1 coordinately activates coactivator(s) of NF- κ B such as members of the Smad family of transcription factor. Indeed, coordinated action of NF- κ B and Smad proteins on their activation by TGF- β 1 has been recently reported (42). Smad proteins, although regarded earlier as quintessential tumor suppressor, have now been

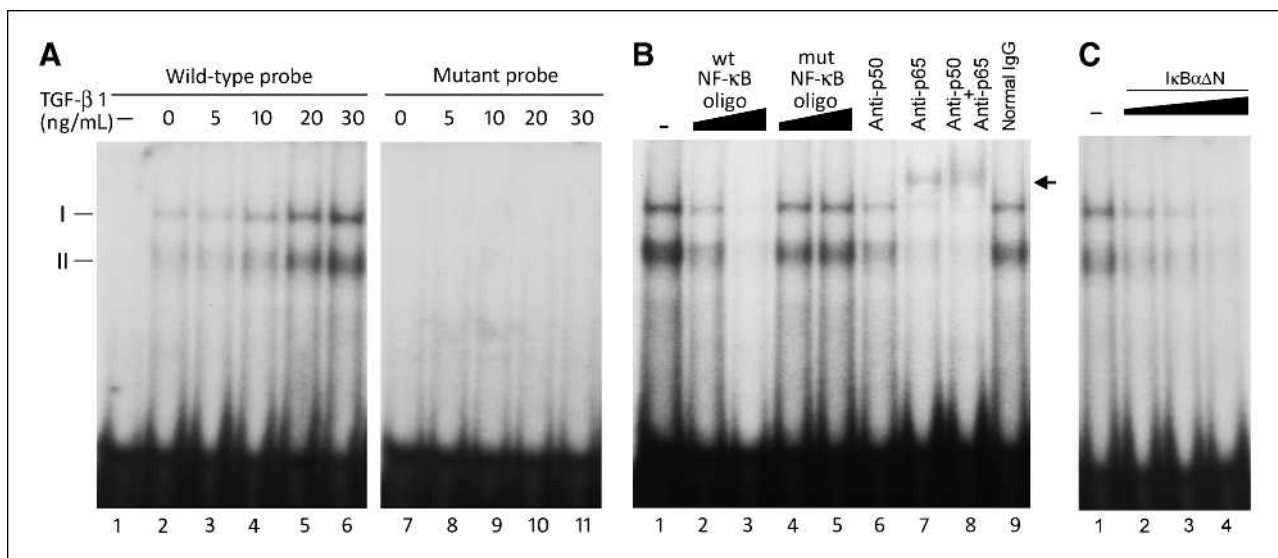


FIGURE 5. Increased interaction of NF- κ B with human ADAM-12 promoter in TGF- β 1-stimulated cells. A, nuclear extracts (10 μ g protein), prepared from MDA-MB-231 cells treated without (lanes 2 and 7) and with various concentrations of TGF- β 1 for 24 h (lanes 3-6 and 8-11), were incubated with a double-stranded ³²P-labeled ADAM-12 DNA probe containing wild-type (lanes 1-6) and mutant (lanes 7-11) NF- κ B elements described in Materials and Methods. Lane 1 contains probe only. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel. Two DNA-protein complexes, I and II, were formed with wild-type probe. B, characterization of the DNA-protein complex. Nuclear extracts (10 μ g protein), prepared from MDA-MB-231 cells treated with TGF- β 1 (20 ng/mL) for 24 h, were incubated with ³²P-labeled wild-type ADAM-12 DNA probe containing wild-type NF- κ B element. In addition, lanes 2 and 3 contain 50 and 100 pmol, respectively, of consensus NF- κ B DNA-binding oligonucleotide (wt NF- κ B oligo); lanes 4 and 5 contain 50 and 100 pmol, respectively, of mutant NF- κ B DNA-binding oligonucleotide (mut NF- κ B oligo); and lanes 6 to 9 contain anti-p50, anti-p65, a combination of anti-p50 + anti-p65 NF- κ B, and normal rabbit IgG, respectively. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel. The arrow indicates the supershifted complex. C, MDA-MB-231 cells were transiently transfected without (lane 1) or with a FLAG-tagged IkB α Δ N expression plasmid (lanes 2-4) and further incubated with TGF- β 1 (20 ng/mL) for 24 h. Nuclear extracts (10 μ g protein) prepared from these cells were incubated with a ³²P-labeled ADAM-12 DNA probe containing an NF- κ B element. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel.

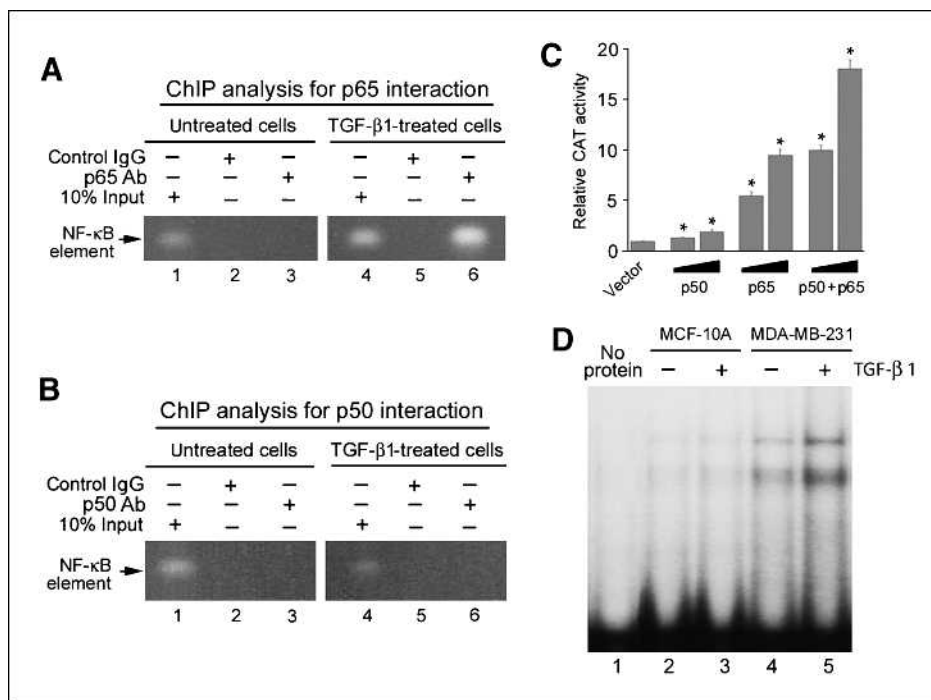


FIGURE 6. *In vivo* recruitment of NF- κ B to the ADAM-12 promoter. **A**, ChIP analysis. MDA-MB-231 cells, treated with or without TGF- β (20 ng/mL) for 24 h, were fixed with formaldehyde, and chromatin prepared from these cells was immunoprecipitated with anti-p65 NF- κ B antibody or with control IgG, as indicated. Specific primer sets were used to amplify the promoter region containing the NF- κ B element in human ADAM-12 promoter. Amplified products were resolved in 1.5% agarose gel. **B**, MDA-MB-231 cells, treated with or without TGF- β (20 ng/mL) for 24 h, were fixed with formaldehyde, and chromatin prepared from these cells was immunoprecipitated with anti-p50 NF- κ B antibody or control IgG, as indicated. Specific primer sets were used to amplify the promoter region encompassing the NF- κ B element in human ADAM-12 promoter. Amplified products were resolved in 1.5% agarose gel. **C**, p50 NF- κ B alone does not transactivate but synergizes p65 NF- κ B-mediated transactivation of human ADAM-12 promoter. MDA-MB-231 cells were cotransfected with 0.6ADAM-12CAT3 reporter (0.5 μ g) and increasing concentrations (0.5 and 1.0 μ g) of expression plasmids of p50, p65, and p50 plus p65 NF- κ B plasmids, or control pcDNA3.1 vector plasmid, as indicated. The amount of plasmid DNA in each transfection assay was kept the same by the addition of carrier plasmid DNA. Induction of CAT activity in equivalent amount of transfected cells was determined and plotted as relative CAT activity. These results represent an average of three independent experiments. *, $P < 0.05$, compared with reporter only-transfected cells. **D**, DNA-binding analysis. Nuclear extracts (10 μ g protein), prepared from MCF-10A (lanes 2 and 3) and MDA-MB-231 (lanes 4 and 5) cells that were treated without (lanes 2 and 4) or with TGF- β 1 (20 ng/mL) for 24 h (lanes 3 and 5), were incubated with a double-stranded 32 P-labeled ADAM-12 DNA probe containing NF- κ B element as described in Materials and Methods. Lane 1 contains probe only. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel.

shown to be prometastatic in the development of breast cancer bone metastasis (43, 44), and these authors have shown that in response to TGF- β 1, the expression of numerous genes, including *interleukin-11* (*IL-11*), is differentially activated in MDA-MB-231 cells compared with MCF-10A cells. Consistent with this finding, TGF- β 1-mediated matrix metalloproteinase-9 induction has been shown to be regulated by NF- κ B, Smad3, and activator protein-1 transcription factors together (45). Although our data show activation of NF- κ B in breast cancer cells and its direct binding to the ADAM-12 promoter (Figs. 3, 5, and 6) and its determining role in promoting ADAM-12 transcription (Figs. 3 and 4), involvement of other factors such as Smad family of proteins in a cooperative fashion with NF- κ B is a possibility. Further studies along this line are currently under way.

Of particular interest in the present study is the direct evidence for TGF- β 1 in inducing human ADAM-12 expression via activation of the p65 NF- κ B subunit. Results of DNA-binding and ChIP analyses (Figs. 5 and 6) clearly

showed the ability of p65 NF- κ B to form specific DNA-protein complex with the κ B element of human ADAM-12 promoter under both *in vitro* and *in vivo* conditions. Interestingly, p50 NF- κ B, which alone exhibited virtually no transactivating action, synergized the action of p65 NF- κ B (Fig. 6). The NF- κ B family is composed of five structurally related proteins that are arranged in two groups (46). The first group containing p65/RelA, RelB, and c-Rel proteins has a well-defined transactivation domain. The other group contains p50 and p52 proteins, which are generated by proteolytic processing of large precursors and lack transactivation domain. The p50 NF- κ B, in general, has been regarded as transcriptionally inactive protein and, in some occasion, is shown to act as a repressor of transcription by occupying the κ B site that could have been interacted by other transcriptionally superior NF- κ B subunits (46).

NF- κ B transcription factor, activated by large number of inflammatory signals, is a regulator of many genes (46). The genes regulated by NF- κ B plays important role in maintaining many aspects of cellular activity, such as

development, immune responses to viral/bacterial infection, cell proliferation-survival-apoptosis, stress, and injury. In all normal cells, with the exception of mature B cells, NF- κ B stays in the cytoplasm in an inactive form through its association with the inhibitory I κ B family of proteins. The inhibitory I κ B α protein is composed of an NH₂ terminus, a central domain containing five ankyrin repeats, and a highly acidic COOH terminus. I κ B α interacts and binds with p65 and p50 subunits of NF- κ B through the ankyrin repeat domains. In response to appropriate stimuli, such as TGF- β , IL-1 β , or tumor necrosis factor- α , I κ B kinase is activated, which in turn phosphorylates I κ B α at NH₂-terminal Ser³² and Ser³⁶. Phosphorylated I κ B α dissociates from the NF- κ B/I κ B α complex and is degraded by the proteasome/ubiquitin pathway, thus allowing activated NF- κ B to translocate to the nucleus (24, 46). Consistent with this pathway, our data show that inhibition of NF- κ B activation by overexpression of phosphorylation-defective and protease-resistant I κ B Δ N protein (24) can block induction of *ADAM-12* promoter-reporter expression by TGF- β 1 (Fig. 3), suggesting a crucial role for active NF- κ B.

In conclusion, this study unveiled a novel mechanism and provides the first evidence for the involvement of NF- κ B in the induction of *ADAM-12* gene expression

and further implicates that this event could be triggered by TGF- β 1 in breast cancer cells. Further understanding of the molecular basis of *ADAM-12* regulation will have functional implications for suppressing extracellular matrix invasion and ultimately lead to design of new therapeutics against cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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