A Three-dimensional Collagen Lattice Activates NF-κB in Human Fibroblasts: Role in Integrin α_2 Gene Expression and Tissue Remodeling

Jiahua Xu,* Mary M. Zutter,‡ Samuel A. Santoro,‡ and Richard A.F. Clark*

*Department of Dermatology, School of Medicine, State University of New York, Stony Brook, New York 11794-8165; and ‡Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. Normal adult human dermal fibroblasts grown in a three-dimensional collagen lattice increase mRNA level of collagen receptor integrin subunit α_2 (Xu, J., and R.A.F. Clark. 1996. *J. Cell Biol.* 132:239–249.) and DNA binding activity of a nuclear transcription factor, NF-κB (Xu, J., and R.A.F. Clark. 1997. *J. Cell Biol.* 136:473–483.). Here we present evidence that the collagen lattice induced the nuclear translocation of p50, one member of NF-κB family, and the degradation of an NF-κB inhibitor protein, IκB-α. The inhibition of NF-κB activity by SN50, a peptide inhibitor targeted at nuclear translocation of NF-κB, significantly reduced the induction of integrin α_2 mRNA and protein by the collagen lattice. A region located between −549 and −351 bp in the promoter of integrin α_2 gene conferred the inducibility by three-dimensional collagen lattice. The presence of either SN50 or IκB-$\alpha^{32, 36}$, a stable mutant of IκB-α, abrogated this inducibility, indicating that the activation of integrin α_2 gene expression was possibly mediated by NF-κB through this region. Although there were three DNA–protein binding complexes forming in this region that are sensitive to the inhibition of NF-κB nuclear translocation, NF-κB was not directly present in the binding complexes. Therefore, an indirect regulatory mechanism by NF-κB in integrin α_2 gene expression induced by three-dimensional collagen lattice is suggested. The involvement of NF-κB in reorganization and contraction of three-dimensional collagen lattice, a process that requires the presence of abundant integrin $\alpha_2\beta_1$, was also examined. The inhibition of NF-κB activity by SN50 greatly blocked the contraction, suggesting its critical role in not only the induction of integrin α_2 gene expression by three-dimensional collagen lattice, but also $\alpha_2\beta_1$-mediated tissue-remodeling process.

Three-dimensional (3D)[1] extracellular matrix (ECM) culture systems have been developed to simulate natural interactions between cells and ECM environment. Fibroblasts cultured in type I collagen matrix can exert forces sufficient to contract the hydrated lattice into dense organized structures resembling dermis. The alignment of collagen fibers that occurs during the embryonic formation of tendons and ligaments and the contraction of collagenous tissue that occurs in healing wounds are thought to be under the influence of the same forces (for review see Grinnell, 1994). Accompanying is the altered expression of a group of genes including integrin α_2 (Klein et al., 1991), type I matrix metalloproteinase (MMP-1; Langholz et al., 1995), and type I collagen (Eckes et al., 1993) in these fibroblasts. Therefore, 3D collagen lattice (COL) may elicit unique signaling processes leading to the altered expression of related genes and the ability of fibroblasts to mediate tissue remodeling.

Integrin $\alpha_2\beta_1$ is a heterodimeric adhesive protein receptor belonging to the β_1 subfamily. It serves as a collagen receptor on fibroblasts and platelets and additionally as a laminin receptor on epithelial and endothelial cells (Elices and Hemler, 1989; Languino et al., 1989; Kirchhofer et al., 1990). Functions mediated by $\alpha_2\beta_1$ may include cell differentiation, motility and metastasis (Chan et al., 1991; Skinner et al., 1994; Keely et al., 1995; Zutter et al., 1995*a*; Doerr and Jones, 1996). For example, the loss of $\alpha_2\beta_1$ in breast epithelial cells is correlated with the transformed phenotype (Keely et al., 1995; Zutter et al., 1995*a*). The interaction of $\alpha_2\beta_1$ integrin with extracellular type I collagen in a 3D polymerized structure has been reported to result

Address all correspondence to Jiahua Xu, Department of Dermatology, School of Medicine, State University of New York, Stony Brook, New York 11794-8165. Tel.: (516) 444-3843. Fax: (516) 444-3844. E-mail: JXu@epo.som.sunysb.edu

1. *Abbreviations used in this paper*: BIM, bisindolylmaleimide GF 109203X; COL, collagen lattice; ECM, extracellular matrix; IκB, inhibitor for NF-κB; IL, interleukin; MMP-1, type I matrix metalloproteinase; NF-κB, nuclear factor κB; PKC, protein kinase C; 3D, three-dimensional; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor.

in the reorganization and contraction of a hydrated collagen matrix (Schiro et al., 1991), and the increased expression of MMP-1 (Riikonen et al., 1995*a*).

The expression of $\alpha_2\beta_1$ is a regulated cellular process. In addition to 3D COL, the regulatory signals for $\alpha_2\beta_1$ integrin expression include PDGF (Ahlen and Kristofer, 1994), TGF-β (Riikonen et al., 1995*b*), EGF (Fujii et al., 1995), PMA (Xu et al., 1996), and oncogenes Erb-B2 and v-ras (Ye et al., 1996). The mechanisms underlying the $\alpha_2\beta_1$ expression stimulated by 3D COL remain unclear. Previously we have presented evidence that a second messenger pathway elicited by 3D COL, which involves protein kinase C–ζ, can mediate integrin α_2 mRNA expression (Xu and Clark, 1997). A recent report showed that other second messenger proteins are modulated by 3D COL such as the suppression of p70 S6 kinase and the elevated levels of $p27^{Kip1}$ and $p21^{Cip1/Waf1}$, inhibitors of cyclin-dependent kinase 2 (cdk2) (Koyama et al., 1996). The 3D COL was also reported to regulate the transcription machinery. For example, the transcription of collagen (Eckes et al., 1993) and albumin (Caron, 1990) genes is downregulated by 3D COL. The DNA binding activity of nuclear transcription factors has been found to be directly regulated by 3D COL. Nuclear extracts of hepatocytes cultured in 3D COL demonstrated induction of DNA binding activity to a TGTTTGC sequence that occurs at regulatory sites of several hepatic genes including albumin, a 3D COL–responsive gene (DiPersio et al., 1991; Liu et al., 1991). We showed previously that fibroblasts cultured in 3D COL increased DNA binding activity of nuclear factor (NF)-κB (Xu and Clark, 1997), a transcription factor that activates gene transcription by binding to a κB sequence motif in the promoter of responsive genes after release from an inactive cytoplasmic complex and translocation to the nucleus (for review see Baeuerle and Baltimore, 1996).

A number of studies have addressed the intimate association between the NF-κB/Rel family of transcription factors and cell adhesion events. First, activation of NF-κB can be caused by the adhesion of cells to fibronectin including human gingival fibroblasts, monkey smooth muscle cells (Qwarnstrom et al., 1994), and human monocytic cell line THP-1 (Lin et al., 1995*a*). The cell-binding domain and the heparin-binding domain of fibronectin molecule were reported to mediate the NF-κB activation (Qwarnstrom et al., 1994). Integrins are considered a critical player in this process since the ligation to β1 integrins to antibody also induces NF-κB activity comparable to cell adhesion to fibronectin (Lin et al., 1995*a*). Second, NF-κB activity is required for the expression of a group of genes encoding cells adhesion molecules such as vascular cell adhesion molecule–1 (VCAM-1), E-selectin, intercellular adhesion molecule–1, and mucosal addressin cell adhesion molecule–1 (for review see Baldwin, 1996). VCAM-1, a cell surface protein typically found on endothelial cells upon stimulation by tumor necrosis factor (TNF)-α, interleukin (IL)-1, or lipopolysaccharide, binds circulating monocytes and lymphocytes expressing $\alpha_4\beta_1$ or $\alpha_4\beta_7$ integrins and likely participates in the recruitment of these cells to sites of tissue injury (Elices et al., 1990; Chan et al., 1992). Additionally, NF-κB seems directly involved in the adhesion of murine embryonic stem cells to various ECM such as gelatin, fibronectin, laminin, and type IV collagen. Antisense RelA oligonucleotides (and in some instances antisense p50 oligonucleotides) administered to various cells, including embryonal stem cells, cause complete detachment from the substratum (Narayanan et al., 1993; Sokoloski et al., 1993). Also, PMA-induced adhesion of HL-60 cells could be inhibited by competitive binding of NF-κB in vivo (Eck et al., 1993).

Little is known about the reciprocal regulation between NF-κB and 3D ECM. In the present study, we characterized the activation of NF-κB and its role in integrin α_2 gene expression by 3D COL. We show in this report that the 3D COL can induce the nuclear translocation of p50, that NF-κB activity was required for the induction of integrin α_2 expression by 3D COL at promoter activity, steady-state mRNA level, and protein level, and that a promoter region conferred 3D COL inducibility. Finally, we demonstrate that NF-κB activity appeared to be required for fibroblast-mediated contraction of 3D collagen matrices.

Materials and Methods

Cell Culture and Reagents

Human fibroblast cultures established by outgrowth from healthy human skin biopsies were provided by M. Simon (SUNY, Stony Brook, NY). The cells were maintained in DME (GIBCO-BRL, Gaithersburg, MD), supplemented with 10% FCS (Hyclone, Logan, UT), 100 U/ml penicillin, 100 U/ml streptomycin (GIBCO-BRL), and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells between population doubling levels 15 and 20 (the 6th and 10th passage) were used for the experiments. Bisindolylmaleimide GF 109203X (BIM) was purchased from CalBiochem-Novabiochem Corp. (La Jolla, CA). SN50 was obtained from BIOMOL (Plymouth Meetings, PA). The control peptide of SN50, SM, was synthesized by Research Genetics Inc. (Huntsville, AL) based on published sequences (Lin et al., 1995*b*). Polyclonal antibodies against NF-κB p65, p50, c-Rel, RelB, Iκ-Bα, and monoclonal antibody against Sp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against β-tubulin and human integrin α_2 subunit were purchased from Chemicon International, Inc. (Temecula, CA). [^{14}C]Chloramphenical, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from DuPont-NEN (Boston, MA).

Plasmids and Oligonucleotides

Plasmids basicCAT and cytomegalovirus-CAT have been described previously (Zutter et al., 1994). Plasmids RSV–β-galactosidase, human α_2 cDNA probe, wild-type IκB-α, and constitutive IκB-$\alpha^{32, 36}$ were provided by L. Taichman (SUNY), Y. Takada (The Scripps Institute, La Jolla, CA) (Takada and Hemler, 1989), W. Greene (The Gladstone Institute of Virology and Immunology), respectively. Human MMP-1 cDNA and α_5 cDNA were purchased from American Type Culture Collection (Rockville, MD) and GIBCO-BRL, respectively. An oligonucleotide complementary to 28S ribosomal RNA was purchased from CLONTECH (Palo Alto, CA). NF-κB and Sp1 enhancer element consensus sequences 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-ATT CGA TCG GGG CGG GGC GAG C-3′, respectively, were purchased from Promega Corp. (Madison, WI). The four repeats of NF-κB consensus element (GGGACTTTCC) as well as four repeats of its mutated sequence (AT-CACTTTCC; mutated bases are underlined) were synthesized and inserted, respectively, into the BamHI site of a promoter-CAT vector purchased from Promega Corp. The construction of a series of α_2CAT plasmids has been described previously (Zutter et al., 1994). For the opposite orientation of an upstream sequence of α_2 promoter (−549 through −351), the DNA fragment was digested with restriction enzymes (BglII and SmaI), filled in at 3′ end, and ligated back into the vector. Deletion mutant, p$\alpha_2$549.dis, was made by restriction enzyme digestion of fragments (−351 through −122 by SmaI and SacII) from p$\alpha_2$549CAT. A second deletion mutant, p$\alpha_2$549.del, was made by removing a fragment (−549 though −351) from p$\alpha_2$776CAT by BglII and SmaI followed by filling in and ligation. For SV-40 promoter–CAT constructs, the α_2 promoter fragment (−549 through −351) in both orientations was inserted into the

BamHI site of the vector. The correctness of the recombinant DNA work was confirmed through restriction digest analysis. The PCR protocol for generating promoter fragments used in DNA–protein binding reaction was as follows: 20 ng of DNA template was amplified at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 30 s in a programmable thermal controller (PTC-100, Model 60; MJ research, Watertown, MA). The sequences of primers used are: pair 1 (5′-$^{-590}$GTATTGCTTAAATATCA^{-573}-3′; 5′-$^{-514}$AGGTTAGAAACTAACTC^{-531}-3′); pair 2 (5′- $^{-516}$CTGGTCATTCTGCGCTTA^{-498}-3′; 5′- $^{-413}$CACTAGCCCTAAACCACA^{-431}-3′); and pair 3 (5′- $^{-415}$GTGCCCTCGGACCCCGCT^{-397}-3′; 5′- $^{-342}$AGTCCCCGGGAGAACGTG^{-360}-3′).

Preparation of COLs

Collagen gels were prepared according to a procedure previously described (Xu and Clark, 1996). Pepsin-solubilized bovine dermal collagen dissolved in 0.012 M HCl was 99.9% pure containing 95–98% type I collagen and 2–5% type III collagen (Vitrogen 100; Celltrix Laboratories, Palo Alto, CA). Collagen for cultures was prepared by mixing 2.0 mg/ml of type I collagen, 100 U/ml penicillin, 100 U/ml streptomycin, and 1% FCS in DME, pH 7.0–7.4. Human dermal fibroblasts from subconfluent cultures starved in 1% FCS for 24 h were mixed with collagen solution for a final concentration of 5×10^5 cells/ml. The collagen cell suspension (4 ml) was immediately placed onto 2% BSA-coated (ICN Biomedicals, Aurora, OH) between, 60-mm petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) and incubated at 37°C for 2 h or for the time periods described in figure legends before the addition of 5 ml of 1% FCS/DME to each dish. In most experiments described here, cells used as control are cultured in 1% FCS/DME on conventional tissue plastic plates.

After incubation at 37°C in 95% air, 5% CO_2, and 100% humidity for the indicated time, cultures were carefully washed twice in DME, and then processed for various analysis. In experiments where inhibitors were used, the levels of lactate dehydrogenase activity released were measured (LD Diagnostic kit; Sigma Chemical Co., St. Louis, MO) and were similar between cells cultured in the presence or absence of inhibitors. When gel contraction experiments were performed, the contraction process was observed and photographed at indicated time points. The surface areas of the gels were measured from prints. Data are presented as relative value and represent 10 individual experiments.

Coating of Petri Dishes

For monolayer collagen coating of plastic dishes, the collagen used for lattices was diluted to a final concentration of 50 μg/ml with PBS. This solution was added to plastic dishes at a final concentration of 6.4 μg/cm^2 and incubated overnight at 4°C. Coated dishes were blocked with 2% BSA for 2 h at room temperature and rinsed with PBS twice before use.

Northern Analysis of Total Cellular RNA

Total RNA was isolated from cell monolayers and collagen gel cultures using a modification of guanidinium thiocyanate method (Chromczynski and Sacchi, 1987). After centrifugation at 14,000 *g* to remove culture medium, collagen gels were dissolved in 4 M guanidinium isothiocyanate and repeatedly passed through a 20.5-gauge needle. For Northern blot hybridization, 3–5 μg of total RNA was treated with glyoxal/DMSO, separated by electrophoresis on an 1% agarose gel in 10 mM phosphate buffer, pH 7.0, and then transferred to Hybond$^+$ nylon membranes (Amersham Corp., Arlington Heights, IL). Ethidium bromide (0.5 μg/ml) was included in the gel to monitor equal loading by the quantity of 18S and 28S ribosomal RNA present. cDNA probes were labeled with [α-^{32}P]dCTP by the random primer procedure (DuPont-NEN, Boston, MA). Oligonucleotide probes were end labeled with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). The filters were hybridized to the labeled probes in QuickHyb solution (Stratagene, La Jolla, CA) for 3 h at 68°C and washed according to manufacturer's protocol. The signals were detected by autoradiography (X-Omat AR; Eastman Kodak, Rochester, NY) at −80°C for optimal exposure. All results shown are representative of at least two independent experiments.

Western Immunoblotting

4–7 μg of proteins from cytoplasmic and nuclear fractions prepared as previously described (Xu and Clark, 1997) were separated on SDS–polyacrylmide gel and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The membranes were incubated with a blocking solution containing 2% BSA, 2% horse serum, 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature, and then incubated overnight at 4°C with various primary antibodies: p50, IκBα, β-tubulin, Sp1 and integrin α_2 subunit. For detection with enhanced chemiluminescence (Amersham Corp.), the blot was incubated with HRP-conjugated goat anti–rabbit antibody (1:1,000 dilution; Amersham Corp.) in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature. For detection with alkaline phosphatase, the membrane was sequentially incubated with biotinylated anti–rabbit goat IgG (H+L) (Vector Labs., Inc., Burlington, CA) at 6 μg/ml (1:250 dilution), and alkaline phosphatase at 1:600 dilution, and then visualized by NBT/BCIP.

Gel Mobility Shift Assay

Gel mobility shift assay was performed as previously described (Xu and Clark, 1997). NF-κB and Sp1 recognition sequences 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-ATT CGA TCG GGG CGG GGC GAG C-3′, respectively, were purchased (Promega, Madison, WI). These oligonucleotides as well as PCR-generated DNA fragments were end labled by [γ-^{32}P]ATP. The nuclear extracts (3–5 μg) were incubated with 1 μg poly (dI/dC) (Boehringer Mannheim) and 2 μg BSA (GIBCO-BRL) in a binding buffer (10 mM Tris, pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 10% glycerol, and $1–5 \times 10^4$ cpm end-labeled oligonucleotides) for 20 min at room temperature. The samples were separated on a 5% native polyacrylamide gel in 0.5× TBE buffer (Tris-borate-EDTA). For supershift assays, 2 μl antibodies were added to the reaction mixture containing end-labeled oligonucleotides, and then incubated for additional 30 min at room temperature. The samples were separated on a 3.5% native polyacrylamide gel.

DNA Transfection

DNA transfection was performed as previously described with a few modifications (Xu et al., 1996). Cells were passaged at $5–7 \times 10^5$ per 10-cm plate. Transfection was performed 22–24 h after passage. Cotransfection was performed with either two plasmids, pα_2-CAT and pRSV-βgal control DNA (15 μg each plasmid), or three plasmids, wild-type or constitutive IκB-α$^{32, 36}$, pα_2–CAT, and pRSV–βgal control DNA (10 μg each), in 1 ml 0.25 M $CaCl_2$ were added dropwise to 1 ml 2× HEBS (50 mM Hepes, pH 7.05, 280 mM NaCl, 1.5 mM Na_2HPO_4) to form a precipitate. Cells in plates were rinsed twice with DME. Precipitate was added to the plate and incubated for 20 min at 37°C in 5% CO_2. DME containing 10% FBS and 50 mM chloroquine was added to 10 ml. After 4 h, medium was replaced with 0.5% FCS. After 40 h, the transfected cells were trypsinized, washed twice with PBS, and then subcultured into collagen gel or onto either tissue plastic or collagen-coated surface for 18–24 h in 0.5% FCS. Protein extracts were prepared after cells were released from 3D COL (Xu and Clark, 1997), and CAT enzyme activity was analyzed as described previously (Xu et al., 1996).

Results

The 3D COL Induces NF-κB Activity

Previously we observed that 3D COL induces integrin α_2 mRNA expression by activating protein kinase C (PKC)-ζ (Xu and Clark, 1997). PKC-ζ has been shown to play an essential role in activating NF-κB by dominant-negative PKC-ζ blocking TNF-α–stimulated NF-κB activity and constitutively active PKC-ζ activating NF-κB in NIH 3T3 cells (Diaz-Meco et al., 1993, 1994; Dominguez et al., 1993; Folgueira et al., 1996). We also obtained evidence that the DNA binding activity of NF-κB, along with the activation of PKC-ζ and integrin α_2 mRNA expression, is induced by 3D COL (Xu and Clark, 1997). Here we examined whether 3D COL can signal the induction of transactivating activity of NF-κB in human dermal fibroblasts. A κB-CAT reporter plasmid was constructed by inserting four repeats of NF-κB consensus element into an SV-40 promoter CAT reporter plasmid vector. Fibroblasts were trans-

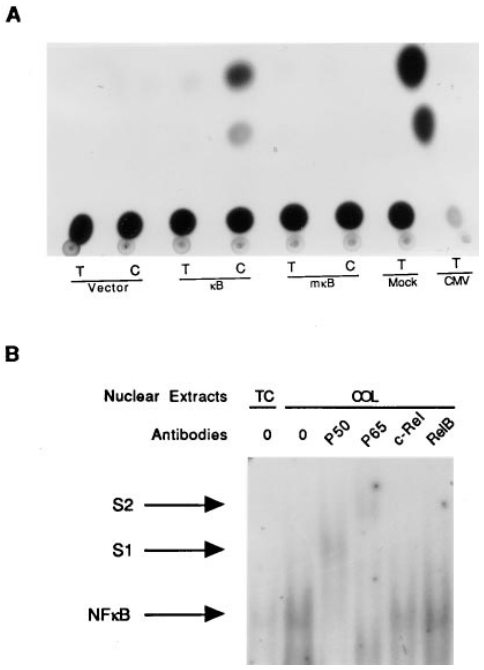

Figure 1. 3D collagen lattice induces NF-κB activation. (*A*) Normal human dermal fibroblasts were transfected with a CAT reporter plasmid that contains 4× κB consensus sequence before stimulation by collagen gel for 18–24 h. CAT activity was assayed and subjected to TLC. *T*, tissue culture; *C*, collagen gel; *Vector*, plasmid without 4× κB; *κB*, plasmid with 4× κB; *mκB*, plasmid with mutated 4× κB. (*B*) Gel mobility supershift assay was performed with nuclear extracts prepared from fibroblasts grown on tissue culture plates (*TC*) or collagen gel (*COL*). The results shown are representative of four independent experiments. *S1*, anti-P50–NF-κB–DNA complex; *S2*, anti-P65–NF-κB–DNA complex. *NF-κB*, (p50-p65)–DNA complex.

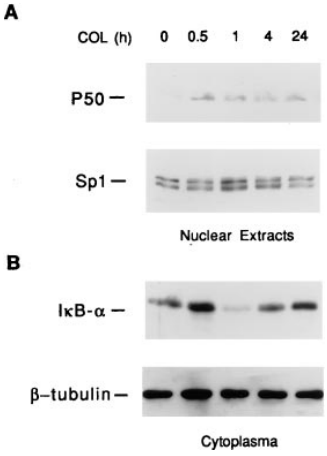

Figure 2. 3D COL induces nuclear translocation of NF-κB and degradation of IκB-α. Nuclear extracts (*A*) and cytoplasmic fractions (*B*) were prepared from normal human dermal fibroblasts grown in collagen gel for indicated length of time. Western analysis was performed with antibodies against p50, Sp1, Iκ-Bα, and β-tubulin. The blots were visualized by enhanced chemiluminescence methods as described in the Materials and Methods. The results are representative of two independent experiments.

fected with the plasmid followed by subculture in 3D COL. As shown in Fig. 1 *A*, 3D COL induced transactivating activity of NF-κB, consistent with the observation made in DNA binding activity (Xu and Clark, 1997). A control plasmid containing the mutant NF-κB recognition sequences did not demonstrate the inducibility by 3D COL (Fig. 1 *A*). The composition of the NF-κB DNA-binding complex was examined next by gel mobility supershift assay. The p50 and p65(RelA) of NF-κB family were found present in the binding complex (Fig. 1 *B*). The composition of the DNA-binding complex remained unchanged during full time-course (24 h) of the induction examined (data not shown).

Although the precise mechanisms underlying the NF-κB induction remain unclear, it is known that the dissociation of NF-κB from IκB, with which it is sequestered as an inactive precursor, and the translocation of NF-κB from cytoplasm to the nucleus are prerequisite for the subsequent nuclear activity of NF-κB. To assess whether nuclear activity of NF-κB observed in Fig. 1 is secondary to its translocation, subcellular fractions from cells cultured in 3D COL were prepared and immunoblotted for p50, one of two subunits that were found in the DNA-binding complex of NF-κB in response to 3D COL (Fig. 1 *B*). The nuclei isolated from cells incubated in 3D COL from 30 min to 24 h showed the presence of p50 in contrast to those from cells grown on tissue culture (Fig. 2 *A*). As a control, Sp1, the transcription factor that binds to its recognition site independent of 3D COL induction (Xu and Clark, 1997), showed the unchanged nuclear protein level during the time-course of incubation in 3D COL (Fig. 2 *A*). The kinetics of protein levels of NF-κB inhibitory protein, IκB, was also examined. Whereas Iκ-Bβ is not detectable in adult dermal fibroblasts (data not shown), Iκ-Bα was detected in the cytoplasmic fractions of quiescent cells (Fig. 2 *B*, lane *1*). The accumulation of Iκ-Bα markedly decreased 1 h after cells were cultured in 3D COL and then resynthesized after 4 h (Fig. 2 *B*). This observation is in accord with the autoregulation of Iκ-Bα by signals that stimulate NF-κB activation via Iκ-Bα degradation (Sun et al., 1993). The detection of β-tubulin in the same sample preparations confirmed the specificity of Iκ-Bα decrease (Fig. 2 *B*). Thus, a correlation was observed between 3D COL stimulation and NF-κB activation, probably by regulating the cellular level of Iκ-Bα.

NF-κB Mediates Integrin α_2 mRNA Expression Stimulated by 3D COL

Next we examined the role of NF-κB in the induction of integrin α_2 mRNA expression by 3D COL. Since p50 responded to a 3D COL signal by undergoing nuclear translocation (Fig. 2 *A*) and forming NF-κB DNA-binding complexes (Xu and Clark, 1997), a cell-permeable synthetic peptide inhibitor, SN50—which carries the hydrophobic region of the signal peptide sequence from Kaposi's fibroblast growth factor (K-FGF) linked to the nuclear localization sequence (NLS) of p50 (Lin et al., 1995*b*)—was used to inhibit NF-κB activity in fibroblasts. SN50 has been reported to specifically inhibit the nuclear translocation of NF-κB in intact cells such as murine endothelial LE-II cells and human monocytic THP-1 cells stimulated by TNF-α and lysophosphatidic acid (LPA) (Lin et al., 1995*b*). The effectiveness of SN50 in inhibiting NF-κB activity stimulated by 3D COL in adult human dermal fibroblasts was examined. As shown in Fig. 3 *A*, NF-κB DNA binding activity was inhibited by the presence of SN50 in a concentration-dependent manner. In contrast, one control peptide, SM, which contains the same signal peptide sequence from K-FGF linked to a random amino acid sequence instead of p50 NLS (Lin et al., 1995*b*), did not show measurable in-

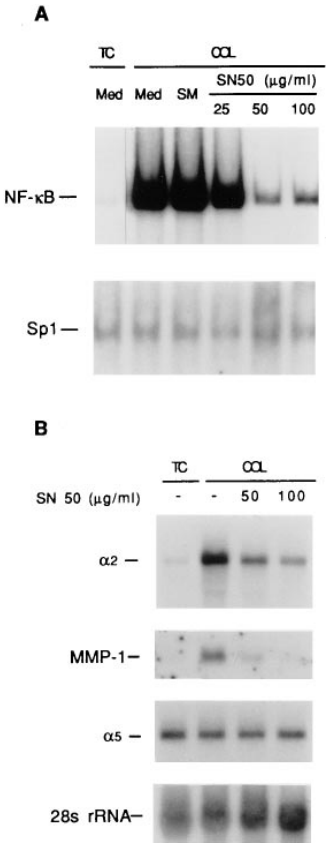

Figure 3. NF-κB mediates induction of integrin α_2 by 3D COL. (*A*) Gel mobility shift assay with nuclear extracts prepared from fibroblasts pretreated with SN50 or SM for 30 min, and subcultured in 3D COL for 20 h in the presence or absence of SN50. Labeled oligonucleotide probes are the consensus sequences for NF-κB and Sp1. (*B*) Fibroblasts were cultured under the same condition as *A*. Total RNA was isolated and subjected to Northern blotting analysis. The results represent two independent experiments. *SN50*, an inhibitor for NF-κB nuclear translocation; *SM*, a control peptide for SN50.

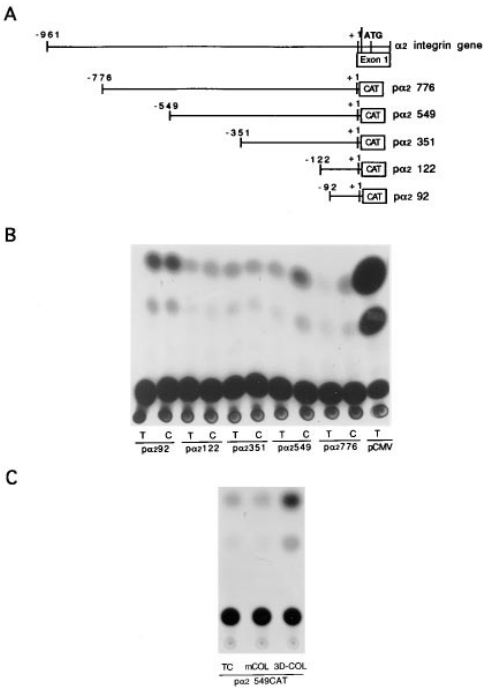

Figure 4. Effect of 5′ deletion on the expression of integrin α_2-CAT fusion gene induced by 3D COL. (*A*) The 5′ flanking region of integrin α_2 gene fused to the CAT structural gene. These constructs were deletion mutants derived from a p$\alpha_2$961-CAT (Zutter et al., 1994). (*B*) Fibroblasts were cotransfected with the 5′ deletion mutant-CAT and RSV–β-galactosidase fusion genes followed by subculture in 3D COL as described in the Materials and Methods. (*C*) Fibroblasts were cotransfected with p$\alpha_2$549-CAT and RSV–β-galactosidase fusion genes followed by subculture on tissue culture plastic plates (*TC*), collagen-coated surface (*mCOL*), and 3D COL. Cells were harvested 1 d after subculture and cell extracts were assayed for CAT activity. The results represent at least five independent experiments. RSV–β-galactosidase activity was used as a control. *pCMV*, cytomegalovirus promoter-CAT construct.

hibitory effects on the DNA binding of NF-κB stimulated by 3D COL (Fig. 3 *A*). To rule out the possibility that the inhibition of NF-κB DNA binding by SN50 is a nonspecific effect we examined the DNA binding ability of the SN50-treated nuclear extracts to Sp1 recognition sequence. The treatment of fibroblasts with SN50 did not prevent Sp1 from binding to its specific sequence (Fig. 3 *A*).

We next measured integrin α_2 mRNA level from cells treated with SN50. The mRNA levels of MMP-1 and integrin α_5 were measured in parallel. The 28S ribosomal RNA was measured as control for gel loading. SN50 inhibited the mRNA levels of integrin α_2 and MMP-1, but not integrin α_5 (Fig. 3 *B*). These results suggest that NF-κB plays an important role in the regulation of integrin α_2 and MMP-1 expression by 3D COL.

3D COL Induces the Promoter Activity of Integrin α_2 Gene

Since NF-κB is a transcription factor, the requirement of its activity in 3D COL stimulation of integrin α_2 mRNA expression indicates that 3D COL may control the transcription of integrin α_2 gene. To assess this possibility, we examined whether 3D COL regulates the promoter activity of integrin α_2 gene. The promoter region of the integrin α_2 gene was cloned and a series of deletion mutants of 5′ flanking sequence were inserted into a CAT reporter vector (Fig. 4 *A*) (Zutter et al., 1994, 1995*b*). Fibroblasts were transiently transfected with these CAT reporter constructs followed by incubation in 3D COL or on two-dimensional surfaces. As shown in Fig. 4 *B*, the reporter gene directed by sequences upstream of −92 bp of integrin α_2 promoter (p$\alpha_2$122CAT, p$\alpha_2$351CAT, p$\alpha_2$549CAT, and p$\alpha_2$776CAT) showed inhibited basal expression when compared to the activity of p$\alpha_2$92CAT (Fig. 4 *B*), indicating the presence of potential silencer element(s). Upon stimuilation by 3D COL, the upstream sequences up to −351 bp of integrin α_2 promoter (p$\alpha_2$92CAT, p$\alpha_2$122CAT, and p$\alpha_2$351CAT) did not show a positive response. In contrast, the plasmids containing either −549 or −776 bp of upstream sequences (p$\alpha_2$549CAT and p$\alpha_2$776CAT) demonstrated 3D COL inducibility. Therefore a region located between −92 and −122 bp probably has negative regulatory sequences for basal promoter activity, whereas the sequences located between −549 and −351 bp of integrin α_2 promoter appear to modulate positive response to 3D COL. For the convenience of this report, we designate this region $\alpha_2^{549-351}$. To understand whether the positive response to 3D COL is elicited by collagen signals resident in collagen molecules of any form or a special collagen structure, we cultured fibroblasts transfected with p$\alpha_2$549CAT on tissue plastic plates, on monolayer collagen-coated plates, and in 3D COL. Cells grown on collagen-coated plates failed to induce reporter gene activity when compared to cells grown on

plastic surface (Fig. 4 *C*), supporting our previous observations of α_2 mRNA (Xu and Clark, 1997). Therefore signals from collagen in a particular 3D structure seemed responsible for the positive response of α_2 promoter modulated by $\alpha_2^{549–351}$.

To further confirm that the positive response conferred by $\alpha_2^{549–351}$ is an enhancer-like response, we examined $\alpha_2^{549–351}$ based on the classic definition of an enhancer element: orientation- and distance-independent activity. A second set of CAT reporter plasmids were constructed that include inverted orientation of $\alpha_2^{549–351}$ (p$\alpha_2$549.revCAT), the altered distance of $\alpha_2^{549–351}$ relative to the transcription initiation site by deletion of the sequence between −351 and −122 bp from p$\alpha_2$549CAT (p$\alpha_2$549.disCAT), and the deletion of $\alpha_2^{549–351}$ from p$\alpha_2$776CAT (p$\alpha_2$549.delCAT) (Fig. 5 *A*). Fibroblasts transfected with this set of plasmids were compared to those with p$\alpha_2$549 CAT, the plasmid containing natural orientation of $\alpha_2^{549–351}$. 3D COL induced CAT level directed from integrin α_2 promoter region (p$\alpha_2$549) was mediated by $\alpha_2^{549–351}$ since the deletion of this region from p$\alpha_2$776 (p$\alpha_2$549.del) abrogated 3D COL inducibility of the reporter plasmid (Fig. 5 *A*), confirming the inability of p$\alpha_2$351 to respond to 3D COL stimulation (Fig. 4 *B*). The 3D COL response mediated by $\alpha_2^{549–351}$ was also independent of either its orientation or its distance to the transcription initiation site since both reporter plasmids (p$\alpha_2$549.revCAT and p$\alpha_2$549.disCAT) showed the similar 3D COL response compared to natural parental reporter plasmid p$\alpha_2$549CAT (Fig. 5 *A*). Furthermore, the deletion of a region between −351 and −122 in p$\alpha_2$549.disCAT did not restore the basal promoter activity (Fig. 5 *A*), indicating the importance of the region between −92 and −122 in its negative regulation as demonstrated by p$\alpha_2$122 (Fig. 4 *B*).

To test whether $\alpha_2^{549–351}$ alone is sufficient for 3D COL induction, we examined the function of this region in a different promoter context. The $\alpha_2^{549–351}$ was inserted in either natural or inverted orientation into an SV-40 promoter-CAT vector lacking of enhancer elements (Fig. 5 *B*). 3D COL induced CAT activity directed from the SV-40 promoter in the presence of $\alpha_2^{549–351}$ in either orientation, but failed to do so in its absence (Fig. 5 *B*). Taken together, these results indicate that 3D COL regulated integrin α_2 gene expression at transcription level and that a promoter region between −549 and −351 bp was necessary and sufficient for the transcriptional stimulation by 3D COL to occur.

NF-κB Mediates Integrin α_2 Gene Transcription Stimulated by 3D COL

The requirement of the NF-κB for 3D COL induction of α_2 promoter activity was investigated. Fibroblasts transfected with $\alpha_2^{549–351}$-containing plasmid (p$\alpha_2$549CAT) were treated with SN50 or its peptide control, SM. SN50 but not SM, inhibited CAT activity directed by −549 bp of integrin α_2 promoter stimulated by 3D COL (Fig. 6 *A*), consistent with the observation made at mRNA steady-state level (Fig. 3 *B*). A PKC inhibitor, BIM, also inhibited the promoter activity, supporting our previous finding that PKC is required for integrin α_2 mRNA expression induced by 3D COL (Xu and Clark, 1997). To further confirm the

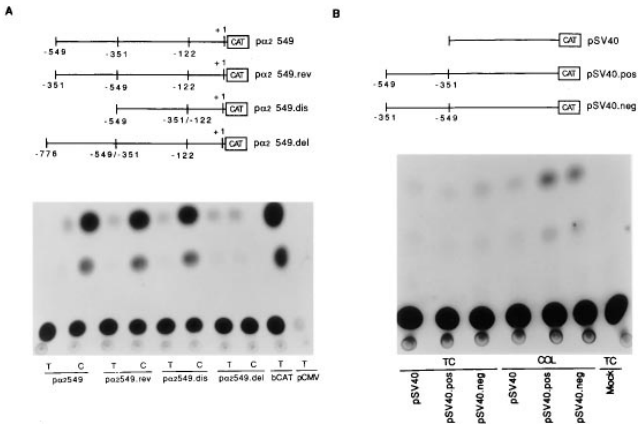

Figure 5. Effects of orientation, altered distance relative to the transcription initiation site, and a different promoter context on the 3D COL indicibility of the upstream sequence from −549 to −351. (*A*) A 5′ flanking region between −549 and −351 bp was rearranged in the p$\alpha_2$549-CAT by inverted orientation (*p$\alpha_2$549.rev*) and different distance relative to the transcriptional initiation site (*p$\alpha_2$549.dis*). The region was deleted from p$\alpha_2$776-CAT (*p$\alpha_2$549.del*). *p$\alpha_2$549.rev*, inverted orientation of the region (−549 through −351) in p$\alpha_2$549-CAT construct; *p$\alpha_2$549.dis*, fusion of the region (−549 through −351) to −122 by deletion of a sequence fragment from −351 to −122 in the p$\alpha_2$549-CAT construct. *p$\alpha_2$549.del*, deletion of the region (−549 through −351) in p$\alpha_2$776-CAT construct. (*B*) A 5′ flanking region spanning between −549 and −351 bp was inserted into an SV-40 promoter-CAT vector in different orientations; *pSV40.pos*, natural orientation of the region (−549 through −351) inserted in SV-40–CAT constructs; and *pSV40.neg*, inverted orientation of the region (−549 through −351) inserted in SV-40–CAT constructs. Fibroblasts were transfected with these constructs and assayed as described in Fig. 4. The results represent at least four independent experiments. *bCAT*, CAT vector that lacks upstream regulatory elements; *pCMV*, cytomegalovirus promoter–CAT construct. *Mock*, plasmid-minus transfection.

inhibitory effects of SN50 on α_2 promoter activity induced by 3D COL, we used a second approach by cotransfection of cells with α_2 reporter plasmids and wild-type IκB-α or a stable mutant IκB-$\alpha^{32, 36}$. Since 3D COL led to IκB-α degradation (Fig. 2 *B*), the IκB-$\alpha^{32, 36}$ mutant, which is resistant to induced degradation (Traenckner et al., 1995), could serve as a potent and specific inhibitor for NF-κB activity (Wang et al., 1996). As shown in Fig. 6 *B*, whereas IκB-$\alpha^{32, 36}$ (IκBDN)-transfected cells did not alter their basal promoter activity as compared with wild-type IκB-α (mock) transfected cells, they demonstrated a drastic reduction in 3D COL–induced p$\alpha_2$549CAT promoter activity. In contrast, the mutant IκB-α did not affect the promoter activity of either p$\alpha_2$92CAT or p$\alpha_2$351CAT when the transfected cells were cultured in 3D COL (Fig. 6 *B*). Therefore it appears that NF-κB transactivating activity was required for the $\alpha_2^{549–351}$-mediated the 3D COL inducibility of integrin α_2 promoter.

Analysis of DNA sequence in this region revealed that a site located between −457 and −447 bp (GGGACGCACC) shares sequence homology but for one base (underlined) to the NF-κB consensus sequence GGGRNNYYCC (R indicates purine; N is any base; Y is pyrimidine) present in a set of inducible genes expressed by human

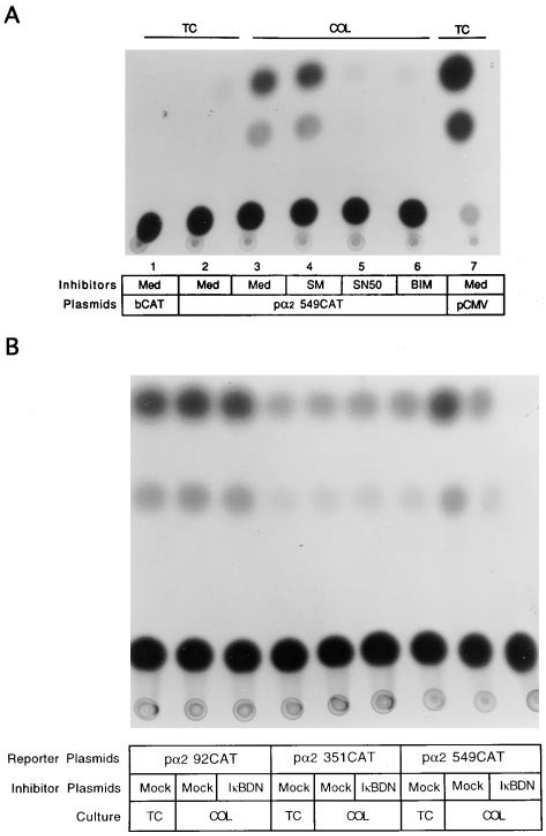

Figure 6. NF-κB mediates induction of integrin α_2 promoter activity by 3D COL. (*A*) Fibroblasts cotransfected with p$\alpha_2$549-CAT and RSV–β-galactosidase fusion gene constructs were pretreated with SN50 or SM for 30 min before stimulation by 3D COL for 18 h. (*B*) Fibroblast were cotransfected with p$\alpha_2$549-CAT, RSV–β-galactosidase fusion gene, and either wild-type IκB-α (*Mock*) or constitutive IκB-α[32, 36] (*IκBαDN*). CAT activity was assayed and subjected to TLC. *BIM*, a protein kinase C inhibitor. The results represent at least four independent experiments.

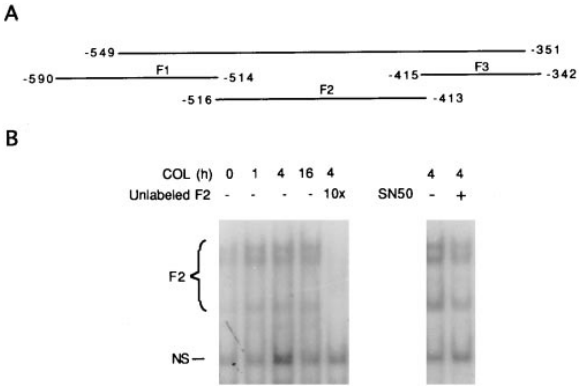

Figure 7. NF-κB mediates DNA–protein binding complex formation on integrin α_2 promoter induced by 3D COL. (*A*) Three DNA fragments were generated by PCR using integrin α_2 promoter as template and used for gel mobility shift assay. *F1*, DNA fragment spanning from −590 to −514 bp; *F2*, DAN fragment spanning from −516 to −413 bp; *F3*, DNA fragment spanning from −415 to −342 bp. (*B*) Nuclear extracts were prepared from fibroblasts grown in 3D COL for the time periods as indicated and from cells grown in 3D COL for 4 h with or without SN50. Gel mobility shift assay was performed using labeled F2, an upstream sequence of integrin α_2 (−516 to −413 bp). Unlabeled F2 was used as DNA competitor. The results represent two independent experiments. *F2*, specific F2–protein complexes; *NS*, nonspecific binding.

monocytic and endothelial cells (Parry and Mackman, 1994). The oligonulceotides synthesized based on this sequence, however, mediated neither DNA–protein complex formation as judged by gel mobility shift assay nor transactivation when inserted into an SV-40 promoter-CAT reporter vector as judged by CAT analysis of transfected cells cultured in 3D COL (data not shown). The observation raised a possibility that α_2 promoter context is required for the detection of NF-κB binding activity to the region. Nuclear protein binding of the entire $\alpha_2^{549–351}$ region was thus examined. Three DNA fragments were generated by PCR: F1, −590 and −514 bp; F2, −516 and −413 bp; and F3, −415 and −342 bp (Fig. 7 *A*). Gel mobility shift assay showed the formation of three specific F2–protein complexes, which were moderately increased by 3D COL (Fig. 7 *B*) whereas F1 and F3 did not demonstrate the specific nuclear protein binding (data not shown). Three approaches were taken to determine whether NF-κB was directly involved in the F2-protein complexes. First, the DNA binding activity of 3D COL–induced nuclear proteins to F2 region of α_2 promoter was reduced to approximately basal level by the incubation of cells with SN50, the p50 nuclear translocation inhibitor (Fig. 7 *B*), suggesting NF-κB activity is required for the binding complex formation. However, the competition with unlabeled NF-κB consensus sequence did not affect the DNA complex formation on F2 (data not shown), suggesting the absence of direct contact of NF-κB with this DNA fragment. Third, supershift assays with antibodies against various proteins of NF-κB family did not yield band shifts (data not shown), supporting the failure of NF-κB to bind this promoter region. Therefore, NF-κB appears to play a critical role in integrin α_2 promoter activation by 3D COL through $\alpha_2^{549–351}$ without direct binding to the region.

NF-κB Mediates Collagen Gel Contraction

It has been reported by various laboratories that integrin $\alpha_2\beta_1$ mediates reorganization and contraction of collagen gels by human cells including fibroblasts (Schiro et al., 1991), cutaneous squamous carcinoma cells (Fujii et al., 1995), retinal pigment epithelial cells (Kupper and Ferguson, 1993), and transformed osteosarcoma cells (Riikonen et al., 1995). It was proposed that stimulants such as EGF (Fujii et al., 1995) and TGF-β (Riikonen et al., 1995*b*) induce 3D COL contraction by increasing integrin $\alpha_2\beta_1$ expression. The transfection of integrin α_2 cDNA into a cell line RD cells that expresses β_1 chain but possess a very low level of $\alpha_2\beta_1$ integrin restores the ability of RD cells to contract collagen gels (Schiro et al., 1991). We hypothesized that since NF-κB mediated α_2 gene expression, it may be in the regulatory pathway leading to α_2-mediated collagen gel contraction. The effects of NF-κB on 3D COL contraction by fibroblasts were examined. Treatment of cells with SN50, but not SM, significantly slowed the contraction process during 72 h examined (Fig. 8, *A* and *B*).

The expression of integrin α_2 protein by fibroblasts cultured in 3D COL was similarly reduced by SN50 but not SM (Fig. 8 *C*). The amount of a nonspecific band of low molecular weight, serving as an internal control, was similar in all conditions (Fig. 8 *C*), confirming the specificity of the inhibtion. Therefore, it further indicates that NF-κB was involved in the integrin α_2 gene expression stimulated by 3D COL and tissue reorganization possibly by maintaining cellular level of integrin α_2.

Discussion

We report here that NF-κB activity mediated the expression of integrin α_2 gene induced by 3D COL and the contraction of 3D COL populated by adult human dermal fibroblasts. The conclusion is supported by four lines of evidence described in this report. First, 3D COL induced nuclear translocation of p50, an NF-κB subunit, and the degradation and resynthesis of IκB-α (Fig. 2, *A* and *B*), an inhibitory protein of NF-κB and an NF-κB-responsive gene product (LeBail et al., 1993; Chiao et al., 1994). Second, two inhibitors of NF-κB activity, a nuclear translocation inhibitor, SN50, and a stable IκB-α mutant, IκB-α[32, 36], both reduced α_2 promoter activity that resides in the upstream region between −549 and −351 bp (Fig. 6, *A* and *B*). Third, SN50 weakened the protein complex formation in an α_2 promoter fragment from −516 to −413 bp (Fig. 7 *B*). Fourth, SN50 reduced both α_2 mRNA (Fig. 3 *B*) and protein levels (Fig. 8 *C*), and slowed down the collagen gel contraction process (Fig. 8 *B*). The observation that 3D COL signaled induction of NF-κB activity is in line of evidence from other laboratories that cell–ECM interactions are associated with activation of NF-κB (Qwarnstrom et al., 1994; Lin et al., 1995*a*; Lofquist et al., 1995) or liver transcription factors (Liu et al., 1991).

The modulation in Iκ-Bα kinetics presents a potentially critical link between cytosolic regulatory events and nuclear transcription in response to 3D COL induction. Like cytokine, phorbol ester, and lipopolysaccharide, which stimulate myeloid, epithelial, and fibroblast cells (Beg and Baldwin, 1993; Brown et al., 1993; Cordle et al., 1993; Henkel et al., 1993; Sun et al., 1993), 3D COL induces the degradation and subsequence resynthesis of Iκ-Bα (Fig. 2 *B*). Thus 3D COL may be listed as another extracellular signal that elicits an array of intracellular signal transmitters leading to posttranslational modification of Iκ-Bα with an eventual consequence of NF-κB activation. Iκ-Bα has been known as a target of many intracellular signals such as tyrosine phosphorylation events (Imbert et al., 1996), the Ras-Raf pathway (Finco and Baldwin, 1993; Li and Sedivy, 1993), PKC-ζ (Diaz-Meco et al., 1994), double-stranded RNA–dependent kinase (Yang et al., 1995), mitogen-activated protein kinase/extracellular response kinase (ERK)-1 (Lee et al., 1997), MAP3K-related kinase (Malinin et al., 1997), and IκB kinase (Zandi et al., 1997). PKC-ζ was reported to be associated with Iκ-Bα phosphorylation and subsequent NF-κB activation (Diaz-Meco et al., 1993; Dominguez et al., 1993; Diaz-Meco et al., 1994; Folgueira, 1996). Along with the evidence in this report that 3D COL modulated cellular level of Iκ-Bα (Fig. 2 *B*), is our previous finding that PKC-ζ activity is induced under the same condition (Xu and Clark, 1997). Therefore, an attractive hypothesis would be that signals from 3D COL are transmitted into the nucleus in part through a PKC-ζ/Iκ-Bα pathway.

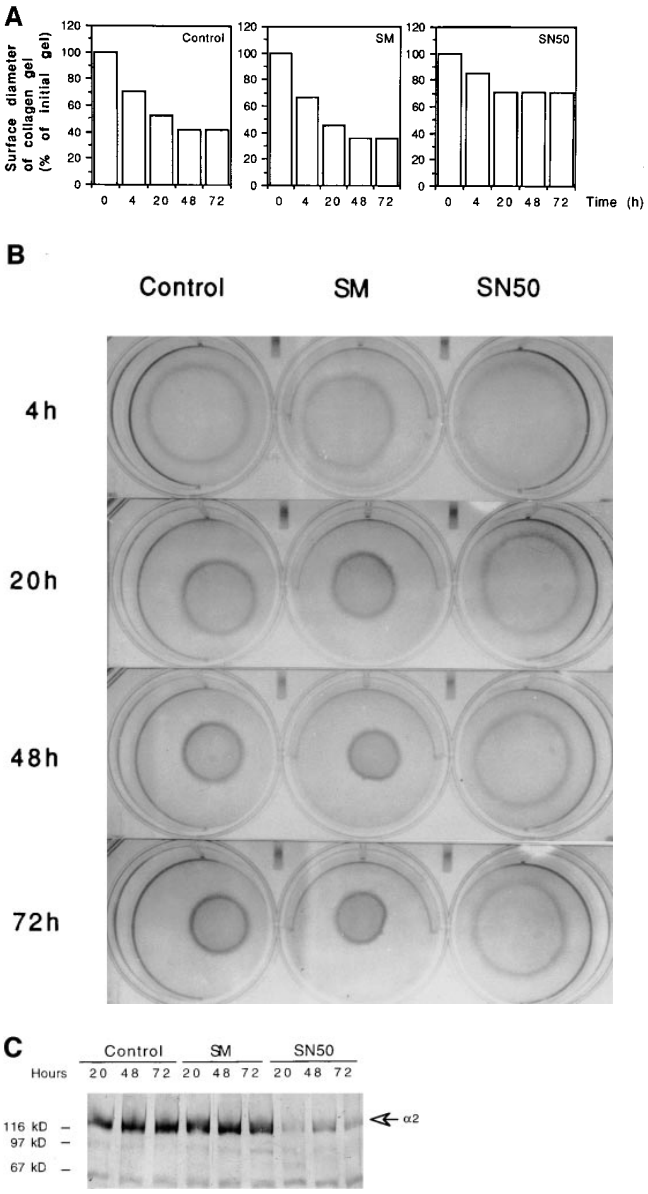

Figure 8. NF-κB mediates 3D COL contraction. Fibroblasts were pretreated with SN50, an inhibitor for NF-κB nuclear translocation, or SM, a peptide control, for 30 min before stimulation by 3D COL. The incubation was continued for 24 h. Cells released from 3D COL by collagenase digestion were subcultured into 3D COL for the measurement of gel contraction in the presence of SN50 or other additives. The surface diameters (*A*) of 3D COLs were measured from the photographs (*B*) taken at 4, 20, 48, and 72 h after subculture. The results represent 10 independent experiments. (*C*) Western analysis of proteins extracted from fibroblasts cultured in 3D COL for 20, 48, and 72 h in the presence of either SM or SN50. The samples were run on 6% SDS–polyacrylamide gel. The blot was detected by an anti-α_2 antibody and visualized with alkaline phosphatase/NBT/BCIP method as described in the Materials and Methods. The integrin α_2 subunit is marked by an arrow. The results represent two independent experiments.

Toward investigating the role of NF-κB in α_2 gene expression, we first questioned whether 3D COL induced α_2

transcription. Although several groups have observed 3D COL induction of α_2 mRNA and/or protein steady-state levels (Klein et al., 1991; Langholz et al., 1995; Xu and Clark, 1996), it was not known whether the regulation occurs at transcriptional level. In this report an upstream region between −549 and −351 bp was identified to confer the 3D COL indicibility of α_2 promoter (Figs. 4 and 5). We confirmed the presence of a general silencer between −92 and −122 bp that suppresses the basal integrin α_2 promoter activity as proposed previously (Zutter et al., 1995*b*). The stimulated expression by 3D COL was mediated by sequences upstream of this region, suggesting that 3D COL could regulate α_2 gene transcription in a DNA sequence–dependent manner, probably by releasing the negative impact of the silencer present between −92 and −122 bp on the promoter activity. A further study of the DNA binding pattern in the 3D COL–responsive region from −549 to −351 bp revealed that there were three DNA sequence–specific protein complexes (Fig. 7). Although the regulation of promoter activity by 3D ECM has not been studied as extensively as those by soluble factors, a similar study on β-casein gene promoter activity conducted by Schmidhauser et al. (1992) identified a 160-bp region in the promoter responsible for its activation by 3D matrigel. In another study, the promoter of TGF-β1 was found to be downregulated by 3D matrigel (Streuli et al., 1993). The involvement of NF-κB activity in α_2 promoter activity was investigated using two inhibitors, SN50, a peptide inhibitor for NF-κB nuclear translocation (Lin et al., 1995*b*), and IκB-$\alpha^{32, 36}$, a stable IκB-α that serves to inhibit NF-κB. Interestingly, whereas NF-κB activity was consistently found to be required for full activation of integrin α_2 expression at levels of mRNA steady-state (Fig. 3 *B*), protein (Fig. 8 *C*), and transcription (Fig. 6), as well as for α_2 promoter–DNA protein complex formation (Fig. 7 *B*), there was no evidence for direct physical involvement of NF-κB in α_2 promoter; this observation is based on competition experiments with unlabeled NF-κB consensus sequence and supershift assay with antibodies against NF-κB proteins (unpublished laboratory data). The observation was further supported by our failure to find any functional activity of a near-perfect NF-κB consensus site located in the region between −549 and −351 bp of α_2 promoter as judged by two sets of experiments: gel mobility shift assay with the synthetic oligonucleotides based on the α_2 NF-κB–like sequence, and functional assay with the site inserted into a reported gene vector (unpublished laboratory data). Taken together, these observations suggest an indirect regulatory mechanism by which NF-κB directs the synthesis of another transcription factor that in turn activates the integrin α_2 promoter. Among many genes known to be mediated by NF-κB are these transcription factors, c-myc (Duyao et al., 1990; LaRosa et al., 1994), interferon regulatory factor 1 (Fujita et al., 1989; Harada et al., 1994), RelA (Ueberla et al., 1993), p50 (Ten et al., 1992), and α-1 acid glycoprotein/enhancer-binding protein (Lee et al., 1996). In fact, we found that 3D COL–induced integrin α_2 mRNA expression requires newly synthesized protein (Xu and Clark, 1997), an observation in accord with the synthesis of an intermediary transcription factor induced by NF-κB. Further studies will be required to address the identity of these three DNA–protein complexes, their regulation by 3D COL, and their functional roles in integrin α_2 gene expression.

The physiological role of NF-κB family proteins is under intensive study. NF-κB has been implicated in biological and pathological processes such as cell death (Grimm et al., 1996; Wu et al., 1996), angiogenesis (Shono et al., 1996), rheumatoid arthritis (Yang et al., 1995), and human cutaneous T lymphoma formation (Thakur et al., 1994). The appropriate regulation of NF-κB activity seems critical for proper biological function. For example, NF-κB activity is required for the H_2O_2-induced tube formation in human microvascular endothelial cells grown on 3D COL (Shono et al., 1996) and antitumor properties of antineoplastic agent taxol in macrophages (Hwang and Ding, 1995). On the other hand, the constitutive NF-κB activation in IκBα$^{-/-}$ transgenic mice causes skin defects (Beg et al., 1995) and severe widespread dermatitis (Klement et al., 1996). The important role of NF-κB in mediating reorganization and contraction of 3D COL as reported here adds another potential biological function of NF-κB. The inhibition of NF-κB activity, which led to inhibited expression of integrin α_2 after 3D COL stimulation, correlated with the inability of cells to contract collagen gel (Fig. 8). NF-κB, therefore, may regulate 3D COL contraction through cell surface expression of $\alpha_2\beta_1$ level. However, it must be noted that other possibilities cannot be ruled out. For example, we found that the presence of $\alpha_2\beta_1$ alone, although neccessary, was not sufficient for the reorganization and contraction of collagen matrices (unpublished laboratory data), suggesting that the availability of $\alpha_2\beta_1$ receptor and the $\alpha_2\beta_1$-elicited second mesenger pathway probably present two separate control levels for 3D COL contraction. Furthermore, the inhibition of NF-κB by SN50 also decreased the 3D COL–induced expression of MMP-1 (Fig. 3 *B*), a protein involved in tissue remodeling. Additionally, NF-κB is strongly implicated in the transcriptional regulation of several growth factors and cytokine genes including interferon-β, IL-1β, IL-2, IL-6, TNF-α and TGF-β1 (for review see Baldwin, 1996), which might offer another interpretation on the mechanism whereby NF-κB modulates tissue remodeling. The essential role for NF-κB in reorganization and contraction of COLs as reported here may present a potentially important nuclear regulatory site for tissue remodeling.

We thank Dr. M. Simon for adult human dermal fibroblasts, Dr. Y. Takada for human α_2 cDNA probe, and Dr. W. Greene for IκB-α plasmids.

Funding for this work was provided by National Institutes of Health grant AG10114309 (R.A.F. Clark). J. Xu was supported by funding from the Dermatology Foundation and the School of Medicine, SUNY at Stony Brook.

Received for publication 17 July 1997 and in revised form 29 November 1997.

References

Ahlen, K., and R. Kristofer. 1994. Platelet-derived growth factor-BB stimulates synthesis of the integrin α_2-subunit in diploid fibroblasts. *Exp. Cell Res.* 215: 347–353.

Baeuerle, P.A., and D. Baltimore. 1996. NF-κB: Ten years after. *Cell.* 87:13–20.

Baldwin, A.S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14:649–681.

Beg, A.A., and A.S. Baldwin, Jr. 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcripion factors. *Genes Dev.* 7:2064–2070.

Beg, A.A., W.C. Sha, R.T. Bronson, and D. Baltimore. 1995. Constitutive NF-κB activation, enhanced granulopoiesis, and neonatal lethality in IκBα-deficient mice. *Genes Dev.* 9:2736–2746.

Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist. 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. *Proc. Natl. Acad. Sci. USA.* 90:2532–2536.

Caron, J.M. 1990. Induction of albumin gene transcription in hepatocytes by extracellular matrix proteins. *Mol. Cell. Biol.* 10:1239–1243.

Chan, B.M., N. Matsuura, Y. Takada, B.R. Zetter, and M.E. Hemler. 1991. In vitro and in vivo consequences of VLA-2 expression on rhabdomyosarcoma cells. *Science.* 251:1600–1601.

Chan, B.M., M.J. Elice, E. Murphy, and M.E. Hemler. 1992. Adhesion of vascular cell adhesion molecule 1 and fibronectin. Comparison of α4 β1 and α4 β7 on the human cell line JY. *J. Biol. Chem.* 267:8366–8370.

Chiao, P.J., S. Miyamoto, and I.M. Verma. 1994. Autoregulation of IκBα activity. *Proc. Natl. Acad. Sci. USA.* 91:28–32.

Chromczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Cordle, S.R., R. Donald, M.A. Read, and J. Hawiger. 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-rel and related NF-κB proteins in human monocytic THP-1 cells. *J. Biol. Chem.* 268:11803–11810.

Diaz-Meco, M.T., E. Berra, M.M. Municio, L. Sanz, J. Lozano, I. Dominguez, V. Diaz-Golpe, M.T.L. de Lera, J. Alcami, C.V. Paya, et al. 1993. A dominant negative protein kinase C ζ subspecies blocks NF-κB activation. *Mol. Cell. Biol.* 13:4770–4775.

Diaz-Meco, M.T., I. Dominguez, L. Sanz, P. Dent, J. Lozano, M.M. Municio, E. Berra, R.T. Hay, T.W. Sturgill, and J. Moscat. 1994. ζ-PKC induces phosphorylation and inactivation of IκB-α in vitro. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2842–2848.

DiPersio, C.M., D.A. Jackson, and K. Zaret. 1991. The extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. *Mol. Cell. Biol.* 11:4405–4414.

Doerr, M.E., and J.I. Jones. 1996. The roles of integrins and extracellular matrix proteins in the insulin-like growth factor 1-stimulated chemotaxis of human breast cancer cells. *J. Biol. Chem.* 271:2443–2447.

Dominguez, I., L. Sanz, F. Arenzana-Seisdedos, M. Diaz-Meco, J.-L. Virelizier, and J. Moscat. 1993. Inhibition of protein kinase C ζ subspecies blocks the activation of an NF-κB-like activity in *Xenopus laevis* oocytes. *Mol. Cell. Biol.* 13:1290–1295.

Duyao, M.P., A.J. Buckler, and G.E. Sonenshein. 1990. Interaction of an NF-κB-like factor with a site upstream of the c-myc promoter. *Proc. Natl. Acad. Sci. USA.* 87:4727–4731.

Eckes, B., C. Mauch, G. Huppe, and T. Krieg. 1993. Downregulation of collagen synthesis in fibroblasts within three-dimensional collagen lattices involves transcriptional and posttranscriptional mechanisms. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 318:129–133.

Elices, M.J., and M.E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. Natl. Acad. Sci. USA.* 86:9906–9910.

Elices, M.J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M.E. Hemler, and R.R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* 60:577–584.

Finco, T.S., and A.S. Baldwin. 1993. κB site-dependent induction of gene expression by diverse inducers of nuclear factor κB requires raf-1. *J. Biol. Chem.* 268:17676–17679.

Folgueira, L., J.A. McElhinny, G.D. Bren, W.S. MacMorran, M.T. Diaz-Meco, J. Moscat, and C.V. Paya. 1996. Protein kinase C-ζ mediates NF-κB activation in human immunodeficiency virus-infected monocytes. *J. Virol.* 70:223–231.

Fujii, K., N. Dousaka-Nakajima, and S. Imamura. 1995. Epidermal growth factor enhancement of HSC-1 human cutaneous squamous carcinoma cell adhesion and migration on type I collagen involves selective up-regulation of $\alpha_2\beta_1$ integrin expression. *Exp. Cell Res.* 216:261–272.

Fujita, T., L.F.L. Reis, N. Watanabe, Y. Kimura, and T. Taniguchi. 1989. Induction of the transcription factor IRF-1 and IFN-κ mRNAs by cytokines and activators of second messenger pathways. *Proc. Natl. Acad. Sci. USA.* 86: 9936–9940.

Grimm, S., M.K.A. Bauer, P.A. Baeuerle, and K. Schulze-Osthoff. 1996. Bcl-2 down-regulates the activity of transcription factor NF-κB induced upon apoptosis. *J. Cell Biol.* 134:13–23.

Grinnell, F. 1994. Fibroblasts, myofibroblasts, and wound contraction. *J. Cell Biol.* 124:401–404.

Harada, H., E.-I. Takahashi, S. Itoh, and K. Harada. 1994. Structure and regulation of the human interferon regulatory factor (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. *Mol. Cell. Biol.* 14: 1500–1509.

Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle. 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature.* 365:182–185.

Hwang, S., and A. Ding. 1995. Activation of NF-κB in murine macrophages by taxol. *Cancer Biochem. Biophys.* 14:265–272.

Imbert, V., R.A. Rupec, A. Livolsi, H.L. Pahl, E.B.-M. Traenckner, C. Mueller, D. Farahifar, B. Rossi, P. Auberger, P.A. Baeuerle, and J.-F. Peyron. 1996. Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. *Cell.* 86:787–798.

Keely, P.J., A.M. Fong, M.M. Zutter, and S.A. Santoro. 1995. Alteration of collagen-dependent adhesion, motility, and morphogenesis by the expression of antisense α_2 integrin mRNA in mammary cells. *J. Cell Sci.* 108:595–607.

Kirchhofer, D., L.R. Languino, E. Ruoslahti, and M.D. Pierschbacher. 1990. $\alpha_2\beta_1$ integrins from different cell types show different binding specifications. *J. Biol. Chem.* 265:615–618.

Kitajima, I., T. Shinohara, J. Bilakovics, D. Brown, X. Xu, and M. Nerenberg. 1992. Ablation of transplanted HTLV-I tax transformed tumors in mice by antisense inhibition of NF-κB. *Science.* 258:1792–1795.

Klein, C.E., D. Dressel, T. Steinmayer, C. Mauch, B. Eckes, T. Krieg, R.B. Bankert, and L. Weber. 1991. Integrin $\alpha_2\beta_1$ is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. *J. Cell Biol.* 115:1427–1436.

Klement, J.F., N.R. Rice, B.D. Car, S.J. Abbondanzo, G.D. Powers, H. Bhatt, C.-H. Chen, C.A. Rosen, and C.L. Stewart. 1996. IκBα deficiency results in a sustained NF-κB response and severe widespread dermatitis in mice. *Mol. Cell. Biol.* 16:2341–2349.

Koyama, H., E.W. Raines, K.E. Bornfeldt, J.M. Roberts, and R. Ross. 1996. Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of cdk2 inhibitors. *Cell.* 87:1069–1078.

Kupper, T.S., and T.A. Ferguson. 1993. A potential pathophysiologic role for $\alpha_2\beta_1$ integrin in human eye diseases involving vitreoretinal traction. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:1401–1406.

Langholz, O., D. Rockel, C. Mauch, E. Kozlowska, I. Bank, T. Krieg, and B. Eckes. 1995. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. *J. Cell Biol.* 131:1903–1915.

Languino, L.R., K.R. Gehlsen, E. Wayner, W.G. Carter, E. Engvall, and E. Ruoslahti. 1989. Endothelial cells use $\alpha_2\beta_1$ integrin as a laminin receptor. *J. Cell Biol.* 109:2455–2462.

LaRosa, F.A., J.W. Pierce, and G.E. Sonensheim. 1994. Differential regulation of the c-myc oncogene promoter by the NF-κB rel family of transcription factors. *Mol. Cell. Biol.* 14:1039–1044.

LeBail, O., R. Schmidt-Ullrich, and A. Israel. 1993. Promoter analysis of the gene encoding the IκB-α/MAD3 inhibitor of NF-κBL positive regulation by members of the rel/NF-κB family. *EMBO (Eur. Mol. Biol. Organ.) J.* 12: 5043–5049.

Lee, F.S., J. Hagler, Z.J. Chen, and T. Maniatis. 1997. Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. *Cell.* 88:213–222.

Lee, Y.-M., L.-H. Miau, C.-J. Chang, and S.-C. Lee. 1996. Transcriptional induction of the α-1 acid glycoprotein (AGP) gene by synergistic interaction of two alternative activator forms of AGP/enhancer-binding protein (C/EBPb) and NF-κB or Nopp140. *Mol. Cell. Biol.* 16:4257–4263.

Li, S., and J.M. Sedivy. 1993. Raf-1 protein kinase activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB-IκB complex. *Proc. Natl. Acad. Sci. USA.* 90:9247–9251.

Lin, T.H., C. Rosales, K. Mondal, J.B. Bolen, S. Haskill, and R.L. Juliano. 1995*a.* Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. *J. Biol. Chem.* 270:16189–16197.

Lin, Y.-Z., S. Yao, R.A. Veach, T.R. Torgerson, and J. Hawiger. 1995*b.* Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J. Biol. Chem.* 270:14255–14258.

Liu, J.-K., C.M. DiPersio, and K.S. Zaret. 1991. Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro. *Mol. Cell. Biol.* 11:773–784.

Lofquist, A.K., K. Mondal, J.S. Morris, and J.S. Haskill. 1995. Transcription-independent turnover of IκBα during monocyte adherence: implications for a translational component regulating IκBα/MAD-3 mRNA levels. *Mol. Cell. Biol.* 15:1737–1746.

Malinin, N.L., M.P. Boldin, A.V. Kovalenko, and D. Wallach. 1997. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature.* 385:540–544.

Narayanan, R., K.A. Higgins, J.R. Perez, T.A. Coleman, and C.A. Rosen. 1993. Evidence for differential function of the p50 and p65 subunits of NF-κB with a cell adhesion model. *Mol. Cell. Biol.* 13:3802–3810.

Parry, G.C., and N. Mackman. 1994. A set of inducible genes expressed by activated human monocytic and endothelial cells contain κB-like sites that specifically bind c-Rel-p65 heterodimers. *J. Biol. Chem.* 269:20823–20825.

Qwarnstrom, E.E., C.O. Ostberg, G.L. Turk, C.A. Richardson, and K. Bomsztyk. 1994. Fibronectin attachment activates the NF-κB p50/p65 heterodimer in fibroblasts and smooth muscle cells. *J. Biol. Chem.* 269:30765–30768.

Riikonen, T., J. Westermarck, L. Koivisto, A. Broberg, V.-M. Kahari, and J. Heino. 1995*a.* Integrin $\alpha_2\beta_1$ is a positive regulator of collagenase (MMP-1) and collagen α1(I) gene expression. *J. Biol. Chem.* 270:13548–13552.

Riikonen, T., L. Koivisto, P. Vihinen, and J. Heino. 1995*b.* Transforming growth factor-β regulates collagengel contraction by increasing $\alpha_2\beta_1$ integrin expression in osteogenic cells. *J. Biol. Chem.* 270:376–382.

Schiro, J.A., B.M.C. Chan, W.T. Roswit, P.D. Kassner, A.P. Pentland, M.E. Hemler, A.Z. Eisen, and T.S. Kupper. 1991. Integrin $\alpha_2\beta_1$ (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell.* 67: 403–410.

Schmidhauser, C., G.F. Casperson, C.A. Myers, K.T. Sanzo, S. Bolten, and M.J.

Bissell. 1992. A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of β-casein gene expression. *Mol. Biol. Cell.* 3:699–709.

Shono, T., M. Ono, H. Izumi, S.-I. Jimi, K. Matsushima, T. Okamoto, K. Kohno, and M. Kuwano. 1996. Involvement of the transcription factor NF-κB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. *Mol. Cell. Biol.* 16:4231–4239.

Skinner, M.P., E.W. Raines, and R. Ross. 1994. Dynamic expression of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin receptors by human vascular smooth muscle cells: $\alpha_2\beta_1$ integrin is required for chemotaxis across type I collagen-coated membranes. *Am. J. Pathol.* 145:1070–1081.

Sokoloski, J.A., A.C. Sartorelli, C.A. Rosen, and R. Narayanan. 1993. Antisense oligonucleotides to the p65 subunit of NF-κB block CD11β expression and alter adhesion properties of differentiated HL-60 granulocytes. *Blood.* 82:625–632.

Streuli, C., C. Schmmidhauser, M. Kobrin, M. Bissell, and R. Derynck. 1993. Extracellular matrix regulates expression of the TGF-β1 gene. *J. Cell Biol.* 120:253–260.

Sun, S.-C., P.A. Ganchi, D.W. Ballard, and W.C. Greene. 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science.* 259:1912–1915.

Takada, Y., and M.E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor α_2 subunit (platelet GPIa): Homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell Biol.* 109:397–407.

Ten, R.M., C.V. Paya, N. Israel, O. LeBail, and M.-G. Matteri. 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:195–203.

Thakur, S., H.C. Lin, W.T. Tseng, S. Kumar, R. Bravo, F. Foss, C. Gelinas, and A. Rabson. 1994. Rearrangement and altered expression of the NFκB-2 gene in human cutaneous T-lymphoma cells. *Oncogene.* 9:2335–2344.

Traenckner, E.B., H.L. Pahl, T. Henkel, K.N. Schmidt, S. Wilk, and P.A. Baeuerle. 1995. Phosphorylation of human IκB on serine 32 and 36 controls IκB proteolysis and NF-κB activation in response to diverse stimuli. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2876–2883.

Ueberla, K., Y.C. Lu, E. Chung, and W.A. Haseltine. 1993. The NF-κB p65 promoter. *J. AIDS Res.* 6:227–230.

Wang, C.-Y., M.W. Mayo, and A.S. Baldwin, Jr. 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science.* 274: 784–787.

Wu, M., H. Lee, R.E. Bellas, S.L. Schauer, M. Arsura, D. Katz, M.J. FitzGerald, T.L. Rothstein, D.H. Sherr, and G.E. Sonenshein. 1996. Inhibition of NF-κB/Rel induces apoptosis of murine B cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:4682–2690.

Xu, J., and R.A.F. Clark. 1996. Extracellular matrix alters PDGF regulation of fibroblast integrins. *J. Cell Biol.* 132:239–249.

Xu, J., and R.A.F. Clark. 1997. A three-dimensional collagen lattice induces protein kinase C-ζ activity: Role in α_2 integrin and collagenase mRNA expression. *J. Cell Biol.* 136:473–483.

Xu, J., M.M. Zutter, S. Santoro, and R.A.F. Clark. 1996. PDGF induction of α_2 integrin gene expression is mediated by protein kinase C-ζ. *J. Cell Biol.* 134: 1301–1311.

Yang, J., J. Merin, T. Nakano, T. Kano, Y. Kitade, and T. Okamoto. 1995. Inhibition of the DNA binding activity of NF-κB by gold compounds in vitro. *FEBS (Fed. Eur. Biol. Soc.) Letts.* 361:89–96.

Yang, Y.L., L.F.L. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B.R.G. Williams, M. Aguet, and C. Weissmann. 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:6095–6106.

Ye, J., R.H. Xu, J. Taylor-Papadimitriou, and P.M. Pitha. 1996. Sp1 binding plays a critical role in Erb-B2- and v-ras-mediated downregulation of α_2-integrin expression in human mammary epithelial cells. *Mol. Cell. Biol.* 16:6178–6189.

Zandi, E., D.M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin. 1997. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell.* 91:243–252.

Zutter, M.M., S.A. Santoro, A.S. Painter, Y.L. Tsung, and A. Gafford. 1994. The human α_2 integrin gene promoter: identification of positive and negative regulatory elements important for cell-type and developmentally restricted gene expression. *J. Biol. Chem.* 269:463–469.

Zutter, M.M., S.A. Santoro, W.D. Staatz, and Y.L. Tsung. 1995*a.* Re-expression of the $\alpha_2\beta_1$ integrin abrogates the malignant phenotype of breast carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 92:7411–7415.

Zutter, M.M., A.A. Painter, W.D. Staatz, and Y.L. Tsung. 1995*b.* Regulation of α_2 integrin gene expression in cells with megakaryocytic features: a common theme of three necessary elements. *Blood.* 86:3006–3014.