# A Three-dimensional Collagen Lattice Activates NF-κB in Human Fibroblasts: Role in Integrin α$_2$ Gene Expression and Tissue Remodeling

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*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-α[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 α$_2$β$_1$, 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 α$_2$β$_1$-mediated tissue-remodeling process.

T HREE-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 α$_2$β$_1$ 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 α$_2$β$_1$ may include cell differentiation, motility and metastasis (Chan et al., 1991; Skinner et al., 1994; Keely et al., 1995; Zutter et al., 1995a; Doerr and Jones, 1996). For example, the loss of α$_2$β$_1$ in breast epithelial cells is correlated with the transformed phenotype (Keely et al., 1995; Zutter et al., 1995a). The interaction of α$_2$β$_1$ integrin with extracellular type I collagen in a 3D polymerized structure has been reported to result

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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., 1995a).

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-$\beta$ (Riikonen et al., 1995b), 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–$\zeta$, can mediate integrin $\alpha_2$ 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)-$\kappa$B (Xu and Clark, 1997), a transcription factor that activates gene transcription by binding to a $\kappa$B 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-$\kappa$B/Rel family of transcription factors and cell adhesion events. First, activation of NF-$\kappa$B 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., 1995a). The cell-binding domain and the heparin-binding domain of fibronectin molecule were reported to mediate the NF-$\kappa$B activation (Qwarnstrom et al., 1994). Integrins are considered a critical player in this process since the ligation to $\beta1$ integrins to antibody also induces NF-$\kappa$B activity comparable to cell adhesion to fibronectin (Lin et al., 1995a). Second, NF-$\kappa$B 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)-$\alpha$, 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-$\kappa$B seems directly involved in the adhesion of murine embryonic stem cells to various ECM such as gelatin, fibronectin, laminin, and type IV collagen. Anti-sense 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-$\kappa$B in vivo (Eck et al., 1993).

Little is known about the reciprocal regulation between NF-$\kappa$B and 3D ECM. In the present study, we characterized the activation of NF-$\kappa$B and its role in integrin $\alpha_2$ gene expression by 3D COL. We show in this report that the 3D COL can induce the nuclear translocation of p50, that NF-$\kappa$B activity was required for the induction of integrin $\alpha_2$ 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-$\kappa$B 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., 1995b). Polyclonal antibodies against NF-$\kappa$B p65, p50, c-Rel, RelB, I$\kappa$-B$\alpha$, and monoclonal antibody against Sp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against $\beta$-tubulin and human integrin $\alpha_2$ subunit were purchased from Chemicon International, Inc. (Temecula, CA). [$^{14}$C]Chloramphenical, [$\alpha$-$^{32}$P]dCTP, and [$\gamma$-$^{32}$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–$\beta$-galactosidase, human $\alpha_2$ cDNA probe, wild-type I$\kappa$B-$\alpha$, and constitutive I$\kappa$B-$\alpha^{32,\ 36}$ 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 $\alpha_5$ 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-$\kappa$B 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-$\kappa$B consensus element (GG-GACTTTCC) 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 $\alpha_2$CAT plasmids has been described previously (Zutter et al., 1994). For the opposite orientation of an upstream sequence of $\alpha_2$ 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 $\alpha_2$ 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 are: pair 1 (5'-$^{-590}$GTATTGCTTAAATATCA$^{-573}$-3'; 5'-$^{-514}$AGGTTAGAAACTAA-CTC$^{-531}$-3'); pair 2 (5'-$^{-516}$CTGGTCATTCTGCGCTTA$^{-498}$-3'; 5'-$^{-413}$CAC-TAGCCCTAAACCACA$^{-431}$-3'); and pair 3 (5'-$^{-415}$GTGCCCTCGG-ACCCCGCT$^{-397}$-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 $5 \times 10^5$ 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/cm² 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 $\alpha_2$ 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 labeled 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, p$\alpha_2$-CAT and pRSV-βgal control DNA (15 μg each plasmid), or three plasmids, wild-type or constitutive IκB-α[32, 36], p$\alpha_2$–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 $\alpha_2$ 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 $\alpha_2$ 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-
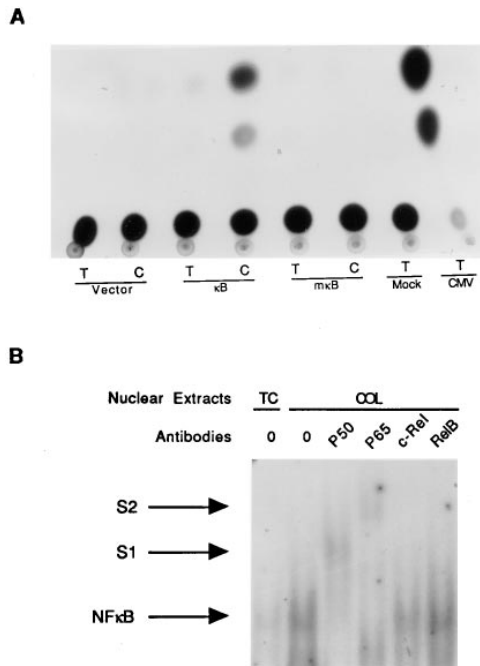
**A**



**B**



*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; *m*κB, 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.

**A**



Nuclear Extracts

**B**



Cytoplasma

*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.

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 α₂ mRNA Expression Stimulated by 3D COL

Next we examined the role of NF-κB in the induction of integrin $\alpha_2$ 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-

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
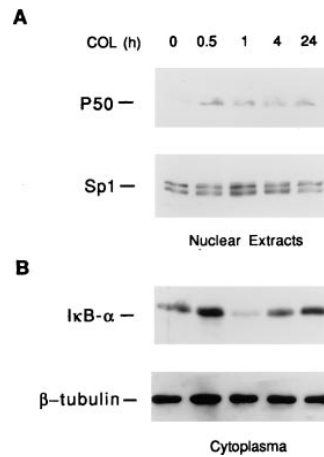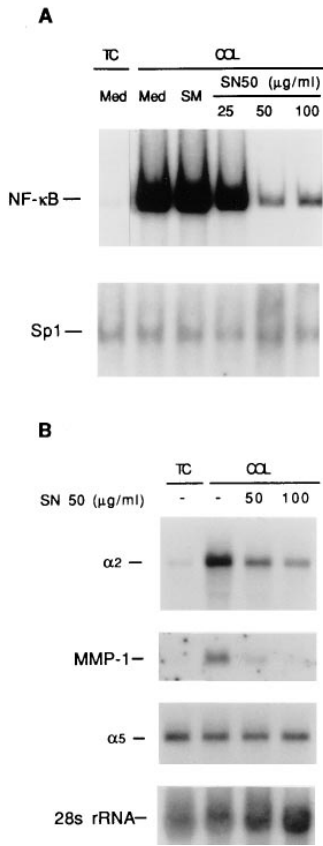
A

| TC | COL | | | | |
|---|---|---|---|---|---|
| Med | Med | SM | SN50 (μg/ml) | | |
| | | | 25 | 50 | 100 |

NF-κB —

Sp1 —

B

| | TC | COL | | |
|---|---|---|---|---|
| SN 50 (μg/ml) | – | – | 50 | 100 |

α2 —

MMP-1 —

α5 —

28s rRNA —

*Figure 3.* NF-κB mediates induction of integrin $\alpha_2$ 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.

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 $\alpha_2$ mRNA level from cells treated with SN50. The mRNA levels of MMP-1 and integrin $\alpha_5$ were measured in parallel. The 28S ribosomal RNA was measured as control for gel loading. SN50 inhibited the mRNA levels of integrin $\alpha_2$ and MMP-1, but not integrin $\alpha_5$ (Fig. 3 *B*). These results suggest that NF-κB plays an important role in the regulation of integrin $\alpha_2$ and MMP-1 expression by 3D COL.

### 3D COL Induces the Promoter Activity of Integrin $\alpha_2$ Gene

Since NF-κB is a transcription factor, the requirement of its activity in 3D COL stimulation of integrin $\alpha_2$ mRNA expression indicates that 3D COL may control the transcription of integrin $\alpha_2$ gene. To assess this possibility, we examined whether 3D COL regulates the promoter activity of integrin $\alpha_2$ gene. The promoter region of the integrin $\alpha_2$ 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 $\alpha_2$ promoter
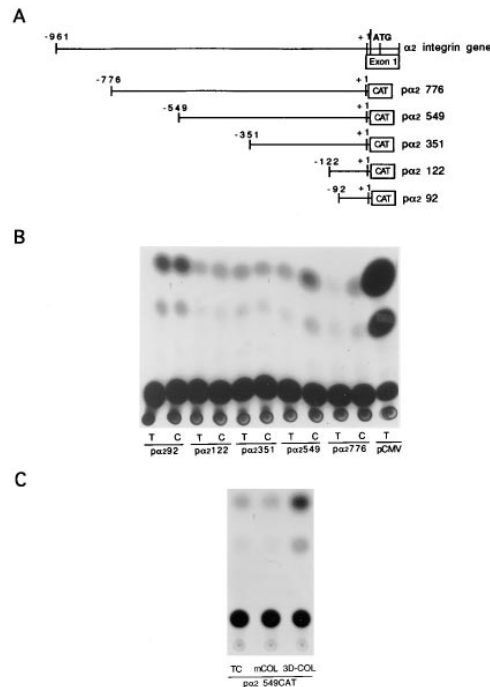


*Figure 4.* Effect of 5' deletion on the expression of integrin $\alpha_2$-CAT fusion gene induced by 3D COL. (*A*) The 5' flanking region of integrin $\alpha_2$ gene fused to the CAT structural gene. These constructs were deletion mutants derived from a p$\alpha_2$961-CAT (Zutter et al., 1994). (*B*) Fibroblasts were cotransfected with the 5' deletion mutant-CAT and RSV–β-galactosidase fusion genes followed by subculture in 3D COL as described in the Materials and Methods. (*C*) Fibroblasts were cotransfected with p$\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.

(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 stimulation by 3D COL, the upstream sequences up to −351 bp of integrin $\alpha_2$ 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 $\alpha_2$ 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 $\alpha_2$ mRNA (Xu and Clark, 1997). Therefore signals from collagen in a particular 3D structure seemed responsible for the positive response of $\alpha_2$ 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.rev-CAT), 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 $\alpha_2$ 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 $\alpha_2$ 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 $\alpha_2$ Gene Transcription Stimulated by 3D COL

The requirement of the NF-κB for 3D COL induction of $\alpha_2$ 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 $\alpha_2$ 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 $\alpha_2$ mRNA expression induced by 3D COL (Xu and Clark, 1997). To further confirm the
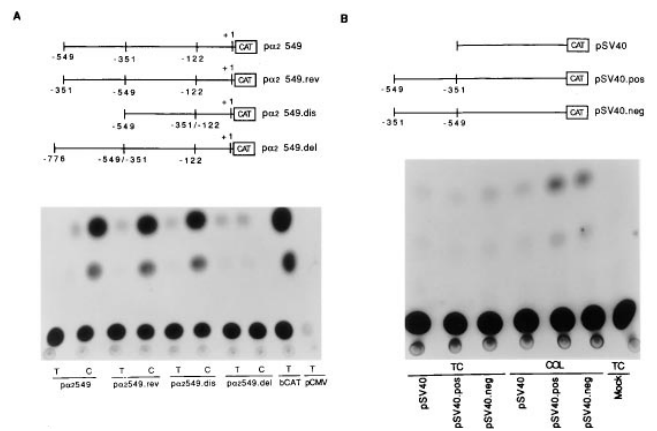


*Figure 5.* Effects of orientation, altered distance relative to the transcription initiation site, and a different promoter context on the 3D COL indicibility of the upstream sequence from $-549$ to $-351$. (*A*) A 5′ flanking region between $-549$ and $-351$ bp was rearranged in the p$\alpha_2$549-CAT by inverted orientation (*p$\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 $\alpha_2$ promoter activity induced by 3D COL, we used a second approach by cotransfection of cells with $\alpha_2$ reporter plasmids and wild-type IκB-α or a stable mutant IκB-α[32, 36]. Since 3D COL led to IκB-α degradation (Fig. 2 *B*), the IκB-α[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-α[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 $\alpha_2$ promoter.

Analysis of DNA sequence in this region revealed that a site located between $-457$ and $-447$ bp (GGGACG-CACC) shares sequence homology but for one base (underlined) to the NF-κB consensus sequence GGGRN-NYYCC (R indicates purine; N is any base; Y is pyrimidine) present in a set of inducible genes expressed by human
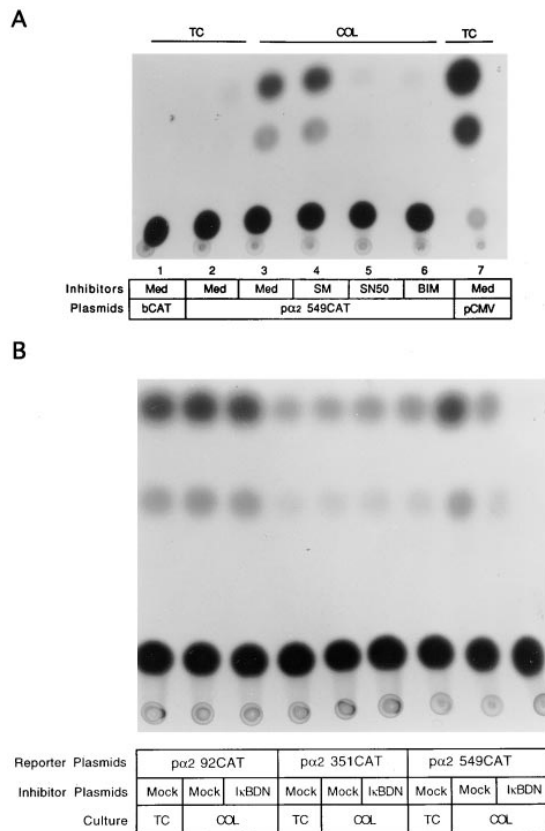
Figure 6. NF-κB mediates induction of integrin $\alpha_2$ promoter activity by 3D COL. (A) Fibroblasts cotransfected with $p\alpha_2549$-CAT and RSV–β-galactosidase fusion gene constructs were pretreated with SN50 or SM for 30 min before stimulation by 3D COL for 18 h. (B) Fibroblast were cotransfected with $p\alpha_2549$-CAT, RSV–β-galactosidase fusion gene, and either wild-type IκB-α (Mock) or constitutive IκB-$\alpha^{32,\,36}$ (IκBαDN). CAT activity was assayed and subjected to TLC. BIM, a protein kinase C inhibitor. The results represent at least four independent experiments.

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 $\alpha_2$ 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 $\alpha_2$ promoter was reduced to approximately basal level by the incubation of cells with SN50, the
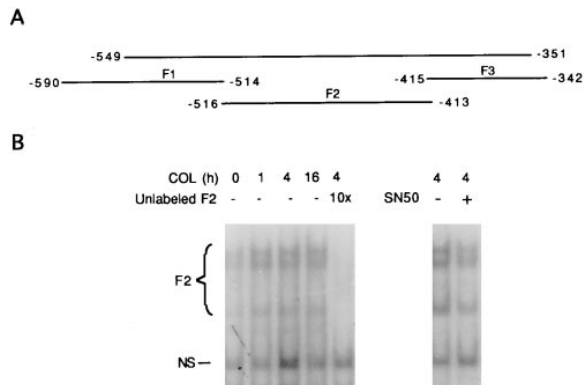


Figure 7. NF-κB mediates DNA–protein binding complex formation on integrin $\alpha_2$ promoter induced by 3D COL. (A) Three DNA fragments were generated by PCR using integrin $\alpha_2$ promoter as template and used for gel mobility shift assay. F1, DNA fragment spanning from −590 to −514 bp; F2, DAN 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 $\alpha_2$ (−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.

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 $\alpha_2$ 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., 1995b) induce 3D COL contraction by increasing integrin $\alpha_2\beta_1$ expression. The transfection of integrin $\alpha_2$ cDNA into a cell line RD cells that expresses $\beta_1$ 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 $\alpha_2$ gene expression, it may be in the regulatory pathway leading to $\alpha_2$-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 $\alpha_2$ 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-$\kappa$B was involved in the integrin $\alpha_2$ gene expression stimulated by 3D COL and tissue reorganization possibly by maintaining cellular level of integrin $\alpha_2$.

## Discussion

We report here that NF-$\kappa$B activity mediated the expression of integrin $\alpha_2$ 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-$\kappa$B subunit, and the degradation and resynthesis of I$\kappa$B-$\alpha$ (Fig. 2, A and B), an inhibitory protein of NF-$\kappa$B and an NF-$\kappa$B-responsive gene product (LeBail et al., 1993; Chiao et al., 1994). Second, two inhibitors of NF-$\kappa$B activity, a nuclear translocation inhibitor, SN50, and a stable I$\kappa$B-$\alpha$ mutant, I$\kappa$B-$\alpha^{32, 36}$, both reduced $\alpha_2$ 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 $\alpha_2$ promoter fragment from $-516$ to $-413$ bp (Fig. 7 B). Fourth, SN50 reduced both $\alpha_2$ 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-$\kappa$B activity is in line of evidence from other laboratories that cell–ECM interactions are associated with activation of NF-$\kappa$B (Qwarnstrom et al., 1994; Lin et al., 1995a; Lofquist et al., 1995) or liver transcription factors (Liu et al., 1991).

The modulation in I$\kappa$-B$\alpha$ 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 I$\kappa$-B$\alpha$ (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 I$\kappa$-B$\alpha$ with an eventual consequence of NF-$\kappa$B activation. I$\kappa$-B$\alpha$ 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-$\zeta$ (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 I$\kappa$B kinase (Zandi et al., 1997). PKC-$\zeta$ was reported to be associated with I$\kappa$-B$\alpha$ phosphorylation and subsequent NF-$\kappa$B 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 I$\kappa$-B$\alpha$ (Fig. 2 B), is our previous finding that PKC-$\zeta$ activity is induced under the same condition (Xu and Clark, 1997). Therefore, an
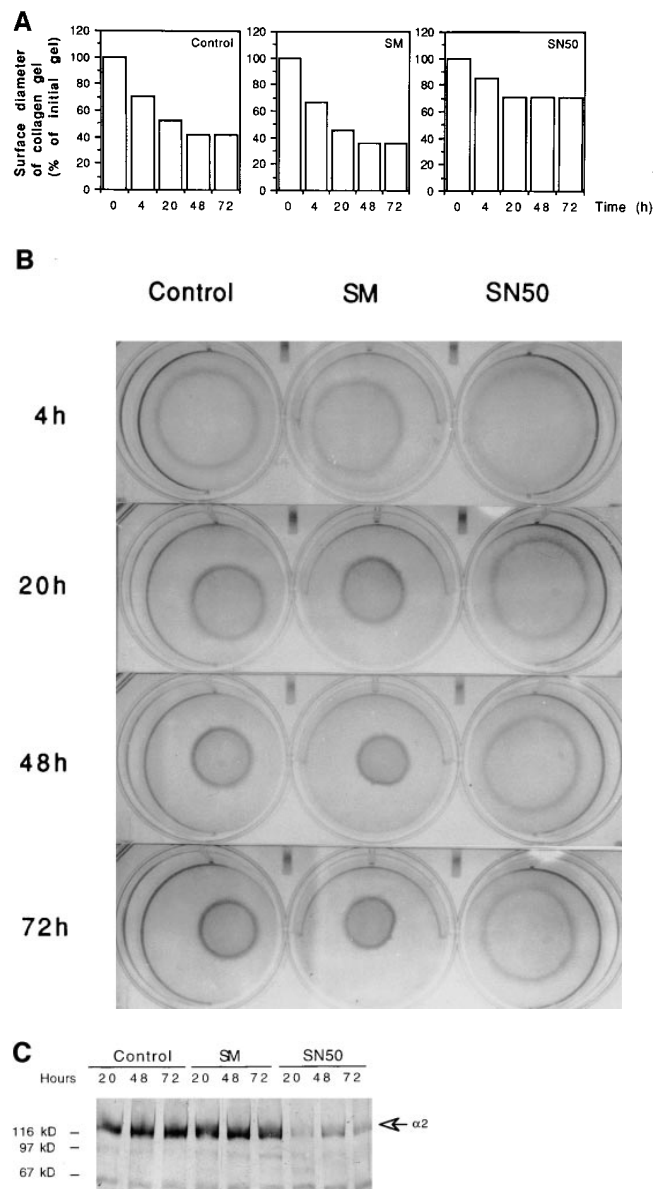


*Figure 8.* NF-$\kappa$B mediates 3D COL contraction. Fibroblasts were pretreated with SN50, an inhibitor for NF-$\kappa$B nuclear translocation, or SM, a peptide control, for 30 min before stimulation by 3D COL. The incubation was continued for 24 h. Cells released from 3D COL by collagenase digestion 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-$\alpha_2$ antibody and visualized with alkaline phosphatase/NBT/BCIP method as described in the Materials and Methods. The integrin $\alpha_2$ subunit is marked by an arrow. The results represent two independent experiments.

attractive hypothesis would be that signals from 3D COL are transmitted into the nucleus in part through a PKC-$\zeta$/I$\kappa$-B$\alpha$ pathway.

Toward investigating the role of NF-$\kappa$B in $\alpha_2$ gene expression, we first questioned whether 3D COL induced $\alpha_2$

transcription. Although several groups have observed 3D COL induction of $\alpha_2$ 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 $\alpha_2$ promoter (Figs. 4 and 5). We confirmed the presence of a general silencer between $-92$ and $-122$ bp that suppresses the basal integrin $\alpha_2$ promoter activity as proposed previously (Zutter et al., 1995b). The stimulated expression by 3D COL was mediated by sequences upstream of this region, suggesting that 3D COL could regulate $\alpha_2$ 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 $\beta$-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-$\beta$1 was found to be downregulated by 3D matrigel (Streuli et al., 1993). The involvement of NF-$\kappa$B activity in $\alpha_2$ promoter activity was investigated using two inhibitors, SN50, a peptide inhibitor for NF-$\kappa$B nuclear translocation (Lin et al., 1995b), and I$\kappa$B-$\alpha$[32, 36], a stable I$\kappa$B-$\alpha$ that serves to inhibit NF-$\kappa$B. Interestingly, whereas NF-$\kappa$B activity was consistently found to be required for full activation of integrin $\alpha_2$ expression at levels of mRNA steady-state (Fig. 3 B), protein (Fig. 8 C), and transcription (Fig. 6), as well as for $\alpha_2$ promoter–DNA protein complex formation (Fig. 7 B), there was no evidence for direct physical involvement of NF-$\kappa$B in $\alpha_2$ promoter; this observation is based on competition experiments with unlabeled NF-$\kappa$B consensus sequence and supershift assay with antibodies against NF-$\kappa$B proteins (unpublished laboratory data). The observation was further supported by our failure to find any functional activity of a near-perfect NF-$\kappa$B consensus site located in the region between $-549$ and $-351$ bp of $\alpha_2$ promoter as judged by two sets of experiments: gel mobility shift assay with the synthetic oligonucleotides based on the $\alpha_2$ NF-$\kappa$B–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-$\kappa$B directs the synthesis of another transcription factor that in turn activates the integrin $\alpha_2$ promoter. Among many genes known to be mediated by NF-$\kappa$B 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 $\alpha$-1 acid glycoprotein/enhancer-binding protein (Lee et al., 1996). In fact, we found that 3D COL–induced integrin $\alpha_2$ 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-$\kappa$B. 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 $\alpha_2$ gene expression.

The physiological role of NF-$\kappa$B family proteins is under intensive study. NF-$\kappa$B 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-$\kappa$B activity seems critical for proper biological function. For example, NF-$\kappa$B 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-$\kappa$B activation in I$\kappa$B$\alpha^{-/-}$ transgenic mice causes skin defects (Beg et al., 1995) and severe widespread dermatitis (Klement et al., 1996). The important role of NF-$\kappa$B in mediating reorganization and contraction of 3D COL as reported here adds another potential biological function of NF-$\kappa$B. The inhibition of NF-$\kappa$B activity, which led to inhibited expression of integrin $\alpha_2$ after 3D COL stimulation, correlated with the inability of cells to contract collagen gel (Fig. 8). NF-$\kappa$B, 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-$\kappa$B by SN50 also decreased the 3D COL–induced expression of MMP-1 (Fig. 3 B), a protein involved in tissue remodeling. Additionally, NF-$\kappa$B is strongly implicated in the transcriptional regulation of several growth factors and cytokine genes including interferon-$\beta$, IL-1$\beta$, IL-2, IL-6, TNF-$\alpha$ and TGF-$\beta$1 (for review see Baldwin, 1996), which might offer another interpretation on the mechanism whereby NF-$\kappa$B modulates tissue remodeling. The essential role for NF-$\kappa$B in reorganization and contraction of COLs as reported here may present a potentially important nuclear regulatory site for tissue remodeling.

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