A Three-dimensional Collagen Lattice Activates NF-κB in Human Fibroblasts: Role in Integrin α2 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 α2 (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 α2 mRNA and protein by the collagen lattice. A region located between −349 and −351 bp in the promoter of integrin α2 gene conferred the inducibility by three-dimensional collagen lattice. The presence of either SN50 or IκB-κ32,36, a stable mutant of IκB-α, abrogated this inducibility, indicating that the activation of integrin α2 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 α2 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 α2β1, 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 α2 gene expression by three-dimensional collagen lattice, but also α2β1-mediated tissue-remodeling process.

THREE-DIMENSIONAL (3D)† 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 α2 (Klein et al., 1991), type I matrix metalloproteinase (MMP-1; Langholz et al., 1995), and type I collagen (Eckes et al., 1991) 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 α2β1 is a heterodimeric adhesive protein receptor belonging to the β1 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 α2β1 may include cell differentiation, motility and metastasis (Chan et al., 1991; Skinner et al., 1994; Keely et al., 1995; Zutter et al., 1995; Dorerr and Jones, 1996). For example, the loss of α2β1 in breast epithelial cells is correlated with the transformed phenotype (Keely et al., 1995; Zutter et al., 1995a). The interaction of α2β1 integrin with extracellular type I collagen in a 3D polymerized structure has been reported to result

† 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 α3β1 is a regulated cellular process. In addition to 3D COL, the regulatory signals for α3β1 integrin expression include PDGF (Ahlen and Kristofer, 1994), TGF-β (Riikonen et al., 1995b), EGF (Fujii et al., 1995), PMA (Xu et al., 1996), and oncopgenes Erb-B2 and v-ras (Ye et al., 1996). The mechanisms underlying the α3β1 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 α3 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 p27Kip1 and p21Cip1/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 TGTTTG 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 monocytes 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-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., 1995a). 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 α6β1 or α5β1 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. Anti-sense RelA oligonucleotides (and in some instances anti-sense p50 oligonucleotides) administered to various cells, including embryonal stem cells, cause complete detachment from the substratum (Narayan 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 α2 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 α2 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% CO2 and 95% air at 37°C. Cells between population doubling levels 15 and 20 (the 6th and 10th passage) were used for the experiments. Bindselymalicemide GF 109203X (BIM) was purchased from CalBiochem-Novabiochem Corp. (La Jolla, CA). SN50 was obtained from BIOLOG (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-κB p65, p50, c-Rel, IκB, and monoclonal antibody against Sp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against β-tubulin and human integrin α2 subunit were purchased from Chemicon International, Inc. (Temecula, CA). 32P]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 α2 cDNA, and wild-type IκBα and constitutive IκBα, 3b 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 α5 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 GGC GAC C-3′, respectively, were purchased from Promega Corp. (Madison, WI). The four repeats of NF-κB element consensus (GG-AGCTTCC) as well as four repeats of its mutated sequence (AT-CAGTTCC, 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 α2CAT plasmids has been described previously (Zutter et al., 1994). For the opposite orientation of an upstream sequence of α2 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, pα−549,dis, was made by restriction enzyme digestion of fragments (−351 through −122 by SmaI and SaclI) from pα,549CAT. A second deletion mutant, pα,549 dél, was made by removing a fragment (−549 though −351) from pα,776CAT by BglII and SmaI followed by filling in and ligation. For SV-40 promoter–CAT constructs, the α2 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’-GGATCCCACTTCATGCTTAGTAAAA-3’); pair 2 (5’-GGATCCATGCTTAGTAAAACCTC-3’); pair 3 (5’-GGATCCCTCGGG-ACCCCGCTCTGGC-3’); pair 4 (5’-GGATCCCTCGGGACCCCGCTCTGGC-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 98.5% 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 × 10⁵ cells/ml. The collagen cell suspension (3 ml) was immediately placed onto 2% BSA-icDNA (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 were used as control unless otherwise indicated. 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 2–5% type III collagen (Vitrogen 100; Celltrix Laboratories, Lincoln Park, NJ) and incubated at 37°C, then processed for Northern blot hybridization. Typically, 5–10 μg of total RNA was hybridized to a 28S ribosomal RNA probe labeled with 32P-ATP. The nucleotide sequence of the 28S ribosomal RNA probe was confirmed through restriction digest analysis. The Northern blot hybridization, autoradiography, and detection were performed according to the instructions provided with the silver-enhanced autoradiography kit (BioRad, Hercules, CA).

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 GGA TCC TTC CCC AGG C-3´ and 5´-ATT CGA TCG GGG CGG GAG C-3´, respectively, were purchased (Promega, Madison, WI). These oligonucleotides as well as PCR-generated DNA fragments were end labeled by [γ-32P]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 7.5, 5 mM MgCl₂, 50 mM KCl, 10% glycerol, and 1–5 × 10⁶ 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 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 × 10⁵ per 10-cm plate. Transfection was performed 22–24 h after passage. Cotransfection was performed with either two plasmids, pAkt-CAT and pRUV-βgal control DNA (15 μg each plasmid), or three plasmids, wild-type or constitutive IκBα-κB, pAkt-CAT, and pRUV-βgal control DNA (10 μg each), in 1 ml 0.25 M CaCl₂ were added dropwise to 1 ml 250 mM chloroquine was added to 1 ml 0.25 M CaCl₂ were added dropwise to 1 ml. After 4 h, medium was replaced with 0.5% FCS. After 24 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 another 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 α₅ 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 α₅ 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-
NF-κB activation, as examined next by gel mobility supershift assay. The p50 as a control, Sp1, the NF-κB–DNA complex; S2, anti-P65–NF-κB–DNA complex. NF-κB, (p50-p65)–DNA complex.

NF-κB Mediates Integrin α2 mRNA Expression Stimulated by 3D COL

Next we examined the role of NF-κB in the induction of integrin α2 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., 1995b)—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., 1995b). 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., 1995b), did not show measurable in-

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, IκB-α, and β-tubulin. The blots were visualized by enhanced chemiluminescence method as described in the Materials and Methods. The results are representative of two independent experiments.
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by sequences upstream of
surfaces. As shown in Fig. 4
followed by incubation in 3D COL or on two-dimensional
transiently transfected with these CAT reporter constructs
flanking sequence were inserted into a CAT reporter vec-
ted the mRNA levels of integrin 
RNA was measured as control for gel loading. SN50 inhib-
ity of integrin 
examined whether 3D COL regulates the promoter activ-
expression indicates that 3D COL may control the tran-
scription of integrin 
plays an important role in the regulation of integrin 
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
results represent two independent experiments. SN50,
an inhibitor for NF-κB nuclear translocation; SM, a control
peptide for SN50.

We next measured integrin α2 mRNA level from cells
treated with SN50. The mRNA levels of MIP-1 and integ-
egrin α5 were measured in parallel. The 28S ribosomal
RNA was measured as control for gel loading. SN50 inhib-
it the mRNA levels of integrin α2 and MIP-1, but not integ-
egrin α5 (Fig. 3 B). These results suggest that NF-κB
plays an important role in the regulation of integrin α2 and
MMP-1 expression by 3D COL.

3D COL Induces the Promoter Activity of
Integrin α2 Gene

Since NF-κB is a transcription factor, the requirement of
its activity in 3D COL stimulation of integrin α2 mRNA
expression indicates that 3D COL may control the tran-
scription of integrin α2 gene. To assess this possibility, we
examined whether 3D COL regulates the promoter activity
of integrin α2 gene. The promoter region of the integ-
egrin α2 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, 1995b). 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 α2 promoter
(pα222CAT, pα2351CAT, pα2549CAT, and pα2776CAT)
showed inhibited basal expression when compared to the
activity of pα92CAT (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 α2
promoter (pα92CAT, pα122CAT, and pα351CAT) did
not show a positive response. In contrast, the plasmids
containing either −549 or −776 bp of upstream sequences
(pα549CAT and pα776CAT) demonstrated 3D COL in-
ducibility. Therefore a region located between −92 and
−122 bp probably has negative regulatory sequences for
basal promoter activity, whereas the sequences located be-
between −549 and −351 bp of integrin α2 promoter appear
to modulate positive response to 3D COL. For the conve-
nience of this report, we designate this region α2−549−351. 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 fi-
broblasts transfected with pα549CAT on tissue plastic
plates, on monolayer collagen-coated plates, and in 3D COL.
Cells grown on collagen-coated plates failed to induce re-
porter gene activity when compared to cells grown on

Figure 3. NF-κB mediates induction of integrin α2 by
3D COL. (A) Gel mobility shift assay with nuclear ex-
tracts prepared from fibroblasts pretreated with SN50
or SM for 30 min, and subcul-
tured in 3D COL for 20 h in
the presence or absence of
SN50. Labeled oligonucle-
otide probes are the consen-
sus sequences for NF-κB and
Sp1. (B) Fibroblasts were
cultured under the same con-
dition as A. Total RNA was
isolated and subjected to
Northern blotting analysis.
The results represent at least five independent experiments. RSV–
β-galactosidase activity was used as a control. pCMV, cytomegalovirus promoter-CAT
construct.

Figure 4. Effect of 5’ deletion on the expression of integrin α2-
CAT fusion gene induced by 3D COL. (A) The 5’ flanking region of
integrin α2 gene fused to the CAT structural gene. These con-
structs were deletion mutants derived from a pα961CAT (Zutter
et al., 1994). (B) Fibroblasts were cotransfected with the 5’ dele-
tion mutant-CAT and RSV–β-galactosidase fusion genes fol-
lowed by subculture in 3D COL as described in the Materials and
Methods. (C) Fibroblasts were cotransfected with pα549-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.
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 $\alpha_2^{549-351}$ ($\text{pa}^{549,\text{rev-CAT}}$), 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 $\text{pa}^{549,\text{CAT}}$ ($\text{pa}^{549,\text{dis-CAT}}$), and the deletion of $\alpha_2^{549-351}$ from $\text{pa}^{776,\text{CAT}}$ ($\text{pa}^{549,\text{del-CAT}}$) (Fig. 5 A). Fibroblasts transfected with this set of plasmids were compared to those with $\text{pa}^{549,\text{CAT}}$, the plasmid containing natural orientation of $\alpha_2^{549-351}$. 3D COL induced CAT level directed from integrin $\alpha_2$ promoter region ($\text{pa}^{549}$) was mediated by $\alpha_2^{549-351}$ since the deletion of this region from $\text{pa}^{776}$ ($\text{pa}^{549,\text{del}}$) abrogated 3D COL inducibility of the reporter plasmid (Fig. 5 A), confirming the inability of $\text{pa}^{549,\text{del}}$ to respond to 3D COL stimulation (Fig. 4 B). The 3D COL response mediated by $\alpha_2^{549-351}$ was independent of either its orientation or its distance to the transcription initiation site since both reporter plasmids ($\text{pa}^{549,\text{rev-CAT}}$ and $\text{pa}^{549,\text{dis-CAT}}$) showed the similar 3D COL response compared to natural parental reporter plasmid $\text{pa}^{549,\text{CAT}}$ (Fig. 5 A). Furthermore, the deletion of a region between $351$ and $122$ in $\text{pa}^{549,\text{dis-CAT}}$ 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 $\text{pa}^{122,\text{CAT}}$ (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.

**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 ($\text{pa}^{549,\text{CAT}}$) 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 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 IkB-α or a stable mutant IkB-α (Wang et al., 1996). Since 3D COL led to IkB-α degradation (Fig. 2 B), the IkB-α (Wang et al., 1996), 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 IkB-α (Wang et al., 1996) (IkBδN)-transfected cells did not alter their basal promoter activity as compared with wild-type IkB-α (mock) transfected cells, they demonstrated a drastic reduction in 3D COL–induced $\alpha_2$ promoter activity. In contrast, the mutant IkB-α did not affect the promoter activity of either $\text{pa}^{549}$ or $\text{pa}^{351}$ 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 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 GGGRRN-NYYCC (R indicates purine; N is any base; Y is pyrimidine) present in a set of inducible genes expressed by human

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**Figure 5. Effects of orientation, altered distance relative to the transcription initiation site, and a different promoter context on the 3D COL inducibility of the upstream sequence from $549$ to $351$.**

(A) A 5′ flanking region between $549$ and $351$ bp was rearranged in the $\text{pa}^{549,\text{rev-CAT}}$ and different distance relative to the transcriptional initiation site ($\text{pa}^{549,\text{dis-CAT}}$). The region was deleted from $\text{pa}^{776}$-CAT ($\text{pa}^{549,\text{del-CAT}}$), $\text{pa}^{549,\text{rev-CAT}}$ and $\text{pa}^{549,\text{dis-CAT}}$. The region ($549$ through $351$) in $\text{pa}^{549,\text{CAT}}$ construct; $\text{pa}^{549,\text{dis-CAT}}$, fusion of the region ($549$ through $351$) to $122$ by deletion of a sequence fragment from $351$ to $122$ in the $\text{pa}^{549,\text{CAT}}$ construct. $\text{pa}^{549,\text{del-CAT}}$, deletion of the region ($549$ through $351$) in $\text{pa}^{776}$-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; $\text{pSV40-CAT}$, natural orientation of the region ($549$ through $351$) inserted in SV-40-CAT constructs; and $\text{pSV40-CAT}$, 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. $\text{bCAT}$, CAT vector that lacks upstream regulatory elements; $\text{pCMV}$, cytomegalovirus promoter–CAT construct. Mock, plasmid-minus transfection.
teins to F2 region of DNA binding activity of 3D COL–induced nuclear protein complexes. First, the approaches were taken to determine whether NF-κB is specifically involved in the F2-protein complexes. Three DNA fragments were generated by PCR using integrin α2 promoter as template and used for gel mobility shift assay. F1, DNA fragment spanning from −590 to −514 bp; F2, DNA 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 α2 (−516 to −413 bp). Unlabeled F2 was used as DNA competitor. The results represent two independent experiments. F2, specific F2–protein complexes; NS, non-specific binding.

NF-κB Mediates Collagen Gel Contraction

It has been reported by various laboratories that integrin αβ1 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 αβ1 expression. The transfection of integrin α2 cDNA into a cell line RD cells that expresses β1 chain but possesses a very low level of α2β1 integrin restores the ability of RD cells to contract collagen gels (Schiro et al., 1991). We hypothesized that since NF-κB mediated α2 gene expression, it may play a critical role in integrin α2 promoter activation by 3D COL through without direct binding to the region. 

Figure 6. NF-κB mediates induction of integrin α2 promoter activity by 3D COL. (A) Fibroblasts cotransfected with pα2,549-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, the 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 α2 promoter induced by 3D COL. (A) Three DNA fragments were generated by PCR using integrin α2 promoter as template and used for gel mobility shift assay. F1, DNA fragment spanning from −590 to −514 bp; F2, DNA 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 α2 (−516 to −413 bp). Unlabeled F2 was used as DNA competitor. The results represent two independent experiments. F2, specific F2–protein complexes; NS, non-specific binding.

monocytic and endothelial cells (Parry and Mackman, 1994). The oligonucleotides 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. It was proposed that CAT analysis of transfected cells cultured in 3D COL (data not shown). The observation raised a possibility that α2 promoter context is required for the detection of NF-κB binding activity to the region. Nuclear protein binding of the entire 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 α2 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 α2 promoter activation by 3D COL through without direct binding to the region.

Xu et al. Integrin Regulation by Extracellular Matrix
The expression of integrin α2 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 inhibition. Therefore, it further indicates that NF-κB was involved in the integrin α2 gene expression stimulated by 3D COL and tissue reorganization possibly by maintaining cellular level of integrin α2.

Discussion

We report here that NF-κB activity mediated the expression of integrin α2 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-α32,36, both reduced α2 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 α2 promoter fragment from −516 to −413 bp (Fig. 7 B). Fourth, SN50 reduced both α2 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., 1995a; Lofquist et al., 1995) or liver transcription factors (Liu et al., 1991).

The modulation in IκB-α 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 subsequent resynthesis of IκB-α (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 IκB-α with an eventual consequence of NF-κB activation. IκB-α 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 IκB-α 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 IκB-α (Fig. 2 B), 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/IκB-α pathway.

Toward investigating the role of NF-κB in α2 gene expression, we first questioned whether 3D COL induced α2
transcription. Although several groups have observed 3D COL induction of α2 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 indiciability of α2 promoter (Figs. 4 and 5). We confirmed the presence of a general silencer between −92 and −122 bp that suppresses the basal integrin α2 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 α2 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 α2 promoter activity was investigated using two inhibitors, SN50, a peptide inhibitor for NF-κB nuclear translocation (Lin et al., 1995b), and IκB-αβ, 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 α2 expression at levels of mRNA steady-state (Fig. 3B) protein (Fig. 8C), and transcription (Fig. 6), as well as for α2 promoter–DNA protein complex formation (Fig. 7B), there was no evidence for direct physical involvement of NF-κB in α2 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 α2 promoter as judged by two sets of experiments: gel mobility shift assay with the synthetic oligonucleotides based on the α2 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 α2 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 α1 acid glycoprotein/enhancer-binding protein (Lee et al., 1996). In fact, we found that 3D COL–induced integrin α2 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 α2 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 H2O2–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 α2 after 3D COL stimulation, correlated with the inability of cells to contract collagen gel (Fig. 8). Moreover, NF-κB, therefore, may regulate 3D COL contraction through cell surface expression of α2β1 level. However, it must be noted that other possibilities cannot be ruled out. For example, we found that the presence of α2β1 alone, although necessary, was not sufficient for the reorganization and contraction of collagen matrices (unpublished laboratory data), suggesting that the availability of α2β1 receptor and the α3β1–elicited second messenger 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. 3B), 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.

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