A Three-dimensional Collagen Lattice Induces Protein Kinase C-ζ Activity: Role in α2 Integrin and Collagenase mRNA Expression

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Abstract. A three-dimensional collagen lattice can provide skin fibroblasts with a cell culture environment that simulates normal dermis. Such a collagen matrix environment regulates interstitial collagenase (type I metalloproteinase [MMP-1], collagenase-1) and collagen receptor α2 subunit mRNA expression in both unstimulated or platelet-derived growth factor–stimulated dermal fibroblasts (Xu, J., and R.A.F. Clark. 1996. J. Cell Biol. 132:239–249). Here we report that the collagen gel can signal protein kinase C (PKC)-ζ activation in human dermal fibroblasts. An in vitro kinase assay demonstrated that autophosphorylation of PKC-ζ immunoprecipitates was markedly increased by a collagen matrix. In contrast, no alteration in PKC-ζ protein levels or intracellular location was observed. DNA binding activity of nuclear factor κB (NF-κB), a downstream regulatory target of PKC-ζ, was also increased by fibroblasts grown in collagen gel. The composition of the NF-κB/Rel complexes that contained p50, was not changed. The potential role of PKC-ζ in collagen gel–induced mRNA expression of collagen receptor α2 subunit and human fibroblast MMP-1 was assessed by the following evidence. Increased levels of α2 and MMP-1 mRNA in collagen gel–stimulated fibroblasts were abrogated by bisindolylmaleimide GF 109203X and calphostin C, chemical inhibitors for PKC, but retained when cells were depleted of 12-myristate 13-acetate (PMA)–inducible PKC isoforms by 24 h of pretreatment with phorbol PMA. Antisense oligonucleotides complementary to the 5’ end of PKC-ζ mRNA sequences significantly reduced the collagen lattice–stimulated α2 and MMP-1 mRNA levels. Taken together, these data indicate that PKC-ζ, a PKC isoform not inducible by PMA or diacylglycerol, is a component of collagen matrix stimulatory pathway for α2 and MMP-1 mRNA expression. Thus, a three-dimensional collagen lattice maintains the dermal fibroblast phenotype, in part, through the activation of PKC-ζ.

The interactions of cells with extracellular matrix (ECM) are essential for cell behavior such as morphology, growth, motility, differentiation, and gene expression. In many biological and pathophysiological processes such as embryonic development, wound healing, tumor invasion and metastasis, and fibrosis, ECM plays this important role not only by its different components but also by its tightly regulated spatial and temporal organizations (Hay, 1991; Lin and Bissell, 1993; Grinnell, 1994; Clark, 1996). Three-dimensional ECM culture systems have been developed to simulate natural interactions between cells and ECM more closely than the traditional in vitro monolayer culture (Grinnell, 1994; Clark et al., 1995; Ronnow-Jensen et al., 1995; Streuli et al., 1995; Sankar et al., 1996). Among those systems, a relaxed collagen lattice populated by fibroblasts is considered an in vitro system representative of a normal fibrous stroma in vivo such as the dermis (Grinnell, 1994). When fibroblasts are embedded in the lattice consisting mainly of type I collagen, they contract the initially loose network to a dense tissue-like structure. This process is accompanied by a fundamental reprogramming of fibroblast morphology and metabolism. This results in down-regulation of type I collagen synthesis (Eckes et al., 1993), attenuation of cellular response to growth factors (Lin and Grinnell, 1993; Clark et al., 1995), induction of collagenase (Unemori and Werb, 1986) and the collagen receptor α2 integrin subunit (Klein et al., 1991), and modulation of platelet-derived growth factor (PDGF) effects on integrin receptor expression (Xu and Clark, 1996).

Much attention has been paid to the role of protein kinase C (PKC) in ECM-regulated cellular activities. A family of serine/threonine-specific protein kinases, PKC has been linked to cell proliferation, differentiation, and regul-
luation of gene expression. This enzyme family can be di-
vided into three groups (for review see Nishizuka, 1995).
The classic group containing isoforms α, β, βII, and γ de-
pends on Ca$^2+$ and phorbol ester/diacylglycerol (DAG) for
activity. The novel group containing isoforms δ, ε, η, θ, and µ is
phorbol ester/DAG-dependent but does not re-
quire Ca$^{2+}$. An atypical group containing PKC-λ, τ, and ζ is
not activated by phorbol ester/DAG. Cell adhesion has
been reported to signal PKC activation. For example, ad-
hesion of HeLa cells to a collagen substratum induces
PKC activity (Chun and Jacobson, 1992, 1993). During
HeLa cell adhesion to a gelatin substratum, PKC-ε is
translocated from cytosolic to membrane fractions (Chun
and Jacobson, 1996). Integrin cell adhesion molecules
could be direct substrates for activated PKC since PKC
phosphorylates the cytoplasmic domain of α6A integrin
subunit in vitro (Gimond et al., 1995). The PKC activity
has been shown to be required for the spreading of several
cell types, such as macrophage on immunoglobulin-coated
surfaces (Li et al., 1996), Hela cells on a collagen substra-
tum (Chun and Jacobson, 1992, 1993), and CHO cells on
fibronectin (Vuori and Ruoslahti, 1993). PKC is also in-
volved in the formation of focal contacts by human embryo
fibroblasts on substrata composed of fibronectin
since its inhibitors reduced focal adhesion and stress fiber
formation (Woods and Couchman, 1992).

The physiological activators of atypical PKC isoforms
are not known. Evidence in the past few years has suggested
that products of phosphoinositol 3-kinase, phosphoinositol
3,4-bisphosphate, and phosphoinositol 3,4,5-trisphosphate
formation (Woods and Couchman, 1992).

Since its inhibitors reduced focal adhesion and stress fiber
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subunit in vitro (Gimond et al., 1995). The PKC activity
increases AA release (Chun and Jacobson, 1992; Chun and
Jacobson, 1996). The PKC activity appears to be a component of the nu-
clear signaling cascade that leads to integrin α2 and MMP-1
expression.

Materials and Methods

Cell Culture

Human fibroblast cultures established by outgrowth from healthy human
skin biopsies were kindly provided by Marcia Simon (Department of Der-
matology, SUNY at Stony Brook). The cells were maintained in DME
(GIBCO BRL, Gaithersburg, MD), supplemented with 10% FCS (Hy-
cel, 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.

Antibodies and Inhibitors

Cycloheximide (CHX) was obtained from Sigma Chemical Co. (St. Louis,
MO). Bisindolylimaleimide GF 109203X (BIM) was purchased from Cal-
biochem-Novabiochem Corp. (La Jolla, CA). Calphostin C and cheleryth-
rine were purchased from LC Laboratories (Woburn, MA). Polyclonal
antibodies against human protein kinase C-δ were purchased from GIBCO BRL.
Monoclonal antibodies against human protein kinase C-ζ, λ and α were purchased from Transduction Laboratories (Lexington,
KY). Polyclonal antibodies against PKC-µ, nuclear factor κB (NF-κB), p65, and p50 were purchased from Santa Cruz Biotechnology (Santa Cruz,
CA). Polyclonal antibodies against human PDGF AB and cyclin A were purchased from Upstate Biotechnology (Lake Placid, NY).

Preparation of Collagen Lattices

Collagen gels were prepared according to a procedure previously de-
scribed (Xu and Clark, 1996). Pepsin-solubilized bovine dermal collagen
in dissolved in 0.012 M HCl was 99.9% pure containing 95–98% type I col-
lagen and 2–5% type III collagen (Vitrogen 100, Cetrix 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
at pH 7.0–7.4. Human dermal fibroblasts from subconfluent cultures
starved in 1% FCS for 24 h were mixed with 10 ml collagen solution for a
final concentration of 5 × 106 cells/ml. The collagen cell suspension (4 ml)
was immediately placed onto 2% BSA (Kankakee, IL)-coated 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 leg-
ends) before the addition of 5 ml of 1% FCS/DME to each dish.

After incubation at 37°C in 95% air; 5% CO2, and 100% humidity for
the indicated time, cultures were carefully washed twice in DME and pro-
cessed for various analyses. In experiments where inhibitors were used,
the levels of lactate dehydrogenase activity released were measured (LD
Diagnostic kit, Sigma Chemical Co.) and found to be similar to cells cul-
tured in the absence of inhibitors.

Coating of Petri Dishes

For monolayer collagen coating of plastic dishes, the collagen used for lat-
tices was diluted to a final concentration of 50 μg/ml with PBS. This solu-
tion 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.

Incubation with Antibodies

Collagen gels minus fibroblasts and 1% FCS/DME were preincubated
with polyclonal antibodies against PDGF AB (100 μg/ml) and, as a con-
trol, cyclin A (100 μg/ml) for 1 h at 4°C. Fibroblasts starved in 1% FCS/
DME for 1 d were detached by trypsinization and then seeded into col-
lagen gels containing relevant antibodies. For antibody blocking of PDGF
stimulation, the medium was replaced with fresh medium containing 30
ng/ml PDGF-BB preincubated with or without anti-PDGF for 1 h.
(PDGF-BB was generously provided by Charles Hart of ZymoGenetics, Se-
attle, WA.) All the control media were replaced accordingly, minus
PDGF-BB. Cells were further incubated for 18 h.

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 me-
dium, collagen gels were dissolved in 4 M guanidinium isothiocyanate and
repeatedly passed through a 20 1/2-gauge needle. For Northern blot hy-
bridization, 3–5 μg of total RNA was treated with glyoxal/DSMO, se-
parated by electrophoresis on an 1% agarose gel in 10 mM phosphate
buffer, pH 7.0, and transferred to Hybond™ nylon membranes (Amer-
sham Corp., Arlington Heights, IL). Ethidium bromide (0.5 μg/ml) was in-
cluded in the gel to monitor equal loading by the quantity of 18 S and 28 S

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ribosomal RNA present. cDNA probes were labeled with [α-32P]dCTP by the random primer procedure (Du Pont/NEB, Boston, MA). Oligonucleotide probes were end-labeled with [γ-32P]ATP (Du Pont/NEB) and in the presence of polyadenylic acid kinase (Boehringer Mannheim Corp., Indianapolis, IN). The filters were hybridized to the labeled probes in QuikHyb solution (Stratagene, La Jolla, CA) for 3 h at 68°C and washed according to manufacturer's protocol. The signals were detected by autoradiography (model X-Omat AR; Kodak Eastman, Rochester, NY) at −80°C for optimal exposure. All results shown are representative of at least two independent experiments. Human α2 cDNA was a generous gift from Dr. Yoshikazu Takada (Scripps Institute, La Jolla, CA) (Takada and Hemler, 1989). Human MMP-1 cDNA was purchased from American Type Culture Collection (Rockville, MD). Human α2 cDNA was purchased from Clontech (Palo Alto, CA).

Preparation of Cell Extracts

Fibroblasts grown in collagen gels were released by digestion of gels with collagenase D (Boehringer Mannheim Corp.). Cells grown on plastic plates were scraped and subsequently processed as those released from collagen gel. Cells from all culture conditions were then washed with ice-cold PBS twice and resuspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, and proteinase inhibitors). After incubation on ice for 30 min, cells were repeatedly passed through a 26 1/2-gauge needle followed by centrifugation at 14,000 g for 20 min. Protein content was determined using a bicinchoninic acid assay (Pierce, Rockford, IL).

Isolation of Nuclei

The nuclei were prepared according to a protocol previously described with some modifications (Greenberg and Ziff, 1984). Cells released from collagen gel or scraped from plastic plates were washed twice with ice-cold PBS. Cells were precipitated by centrifugation at 500 g for 5 min at 4°C. Cell pellet was washed in buffer A once (10 mM Tris, pH 7.4, 3 mM CaCl2, 2 mM MgCl2), resuspended in 1 mL lysis buffer B (0.5% NP-40 in buffer A) and homogenized in a Dounce homogenizer with a “B” pestle. After examination under a microscope to monitor the release of nuclei, the homogenate was centrifuged at 500 g at 4°C. The pellet was designated as nuclei, whereas the supernatant was designated as cytoplasmic fraction. The nuclear pellet was stored in buffer C (50 mM Tris, pH 8.0, 40% glycerol, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM PMSF, 1 μM leupeptin). The protein content in cytoplasm and nuclei was determined using the bicinchoninic acid assay (Pierce).

Western Immunoblotting

Proteins from cell and nuclear extracts were separated on 8% SDS–polyacrylamide gel and transferred to polyvinyl difluoride membranes (Millipore Corp., Bedford, MA). Samples containing 4–7 μg of total proteins were loaded onto the gel. 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, the blots were then visualized with enhanced chemiluminescence (Amersham Corp.).

Immunoprecipitation and In Vitro PKC-ζ Activity Assay

Cell extracts prepared from 106 cells were incubated at 4°C overnight with a rabbit polyclonal antibody against human PKC-ζ used in Western analysis. The immune complexes were recovered by anti-rabbit IgG agarose beads (Sigma Chemical Co.). The resulting immunoprecipitates were washed three times with a cold buffer containing 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, and 25 μg/ml aprotinin before they were mixed in a final volume of 50 μl assay solution (35 mM Tris, pH 7.5, 15 mM MgCl2, 1 mM MnCl2, 0.5 mM EGTA, 0.1 mM CaCl2, 1 mM sodium orthovanadate, and 100 μM [γ-32P]ATP with or without 280 μg/ml phosphorylaseb) and incubated at 30°C for 10 min. After reactions were stopped by the addition of the equal volume of 2× gel loading buffer, the samples were boiled for 3 min and precipitated. The supernatant was analyzed by SDS-PAGE followed by autoradiography.

Gel Mobility Shift Assay

Nuclear extracts were prepared by a modified minieartation protocol (Schreiber et al., 1989). Cells were washed with ice-cold PBS twice and hypotonic buffer A once (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM PMSF, 0.5 mM DTT). Cells were lysed by incubation in lysis buffer B (0.2% NP-40 in buffer A) for 10 min on ice. After centrifugation at 500 g for 4 min at 4°C, the nuclear pellet was resuspended in extraction buffer C (20 mM Tris, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM KCl, 0.5 mM PMSF, 0.5 mM DTT) and D (20 mM Tris, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 1.2 M KCl, 0.5 mM PMSF, 0.5 mM DTT) at a 1:2 ratio followed by incubation on ice for 20 min. The supernatant was collected as nuclear extracts after centrifugation at 14,000 g for 8 min. The nuclear protein was determined using the bicinchoninic acid assay (Pierce).

Gel mobility shift assay was performed with nuclear extracts prepared as described above, NF-xb and Sp-1 enhancer element consensus sequences 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 5'-ATT CGA TCG GGG CCG GAG CAG C-3', respectively, were purchased (Promega Corp., Madison, WI). These oligonucleotides were labeled by [γ-32P]ATP. The nuclear extracts (3–5 μg) were incubated with 1 μg poly (dl/dc) (Boehringer Mannheim Corp.) and 2 μg BSA (GIBCO BRL) in a binding buffer (10 mM Tris, pH 7.9, 5 mM MgCl2, 50 mM KCl, 10% glycerol, and 1–5 × 105 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 assay, the nuclear extracts were incubated with rabbit polyclonal antibody against human PKC-ζ used in Western analysis for 1 h at 4°C. The immune complexes were recovered by anti-rabbit IgG agarose beads followed by centrifugation and then incubated overnight at 4°C with various antibodies: monoclonal PKC-α, , and β- and δ-tubulin, and polyclonal PKC-ζ. After incubation with horseradish peroxidase–conjugated goat anti–rabbit antibody (1:1,000 dilution; Amersham Corp.) in 0.5% Tween 20 in TBS-T (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature, the blots were then visualized with enhanced chemiluminescence (Amersham Corp.).

Down-Regulation of PKC-ζ Protein by Antisense Inhibition of Translation

The procedure was essentially as previously described (Xu et al., 1996) with modifications to accommodate the collagen gel culture. Phosphorothioate DNA oligonucleotides with the sequences 5'-AGTCCAGCAGGAC-3' (sense 1143), 5'-GTCCTCYTGGTGGCAT-3' (antisense 1142), and 5'-GTCCTCTGACGCGGATGACGAAAGC-3' (antisense 1144) were synthesized by Promega. Subconfluent adult human dermal fibroblasts were treated with oligonucleotides in DME containing 20 μg/ml lipofectin (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride [DOTMA]; GIBCO BRL) for 6–8 h at 37°C in the presence of 5% CO2. After this time, the medium containing lipofectin was replaced by fresh medium containing appropriate oligonucleotides. After 48 h, cells were subcultured into collagen gel containing freshly added oligonucleotides. After incubation for 18 h, total RNA or cell extracts were prepared and analyzed with Northern blotting, Western blotting, or immunoprecipitation and kinase assay.

Results

Inhibition of Collagen Gel–induced α2 and MMP-1 mRNA Levels

Previous studies have shown that collagen gel increases α2 integrin mRNA steady-state levels in human foreskin (Klein et al., 1991) and adult dermal (Xu and Clark, 1996) fibroblasts. Relaxed collagen gels also induce collagen gel–induced mRNA (Unemori and Werb, 1986; Langholz et al., 1995). Here we demonstrate that the induction of α2 and MMP-1 mRNA occurred only when cells were cultured in a three-dimensional collagen gel, not on collagen monolayer–coated surface (Fig. 1 A), in agreement with Langholz et al. (1995). A time course showed that the mRNAs appeared as early as 4 h after cells were placed in collagen lattices (Fig. 1 B). α2 mRNA reached the maximum after 24 h, whereas MMP-1 continued to increase up to 72 h (Fig. 1 B).
Since PDGF-BB also induces steady-state levels of $\alpha_2$ mRNA (Ahlen and Rubin, 1994; Xu et al., 1996), we asked whether the induction by collagen gel was caused by PDGF present in collagen preparations. Antibody against PDGF has been shown to neutralize PDGF biological effects (Ferns, 1991). Polyclonal antibody against PDGF AB was therefore included in the collagen gel cultures and, as anticipated, the antibodies did not affect $\alpha_2$ induction, the addition of PDGF cannot further increase $\alpha_2$ expression. Fibroblasts grown in collagen gel were further stimulated by PDGF-BB at 30 ng/ml, and integrin $\alpha_2$ mRNA level by PDGF-BB at 30 ng/ml, and integrin $\alpha_2$ mRNA level was greatly increased compared to COL or PDGF alone (Fig. 1 C, lanes 5 and 9), confirming our previous observation (Xu and Clark, 1996). This further induction was inhibited by anti-PDGF in a dose-dependent manner (Fig. 1 C, lanes 10 and 11). A control antibody, cyclin A, did not have any effect (Fig. 1 C, lane 12). Taken together, signals from three-dimensional collagen lattices responsible for induction of integrin $\alpha_2$ and MMP-1 do not emanate from PDGF contamination.

The requirement of newly synthesized proteins and posttranslational modifications was investigated next. Protein synthesis was required since both types of mRNA diminished in the presence of CHX, a protein synthesis inhibitor (Fig. 2 A). To understand what role PKC might play, three specific PKC inhibitors, BIM, calphostin C (CalC), and chelerythrine, were used in the study. Both BIM and CalC blocked the induction of both mRNAs in a dose-dependent manner, indicating that protein phosphorylation by PKC is required for both $\alpha_2$ and MMP-1 mRNA levels.

**Figure 1.** Northern analysis of integrin $\alpha_2$ subunit and MMP-1 mRNA induced by three-dimensional collagen lattices. Normal human dermal fibroblasts were starved 1 d in 1% FCS/DME before subculture in test conditions. (A) Cells were subcultured on tissue culture plates (TC), collagen monolayer coated surface (ML), and in three-dimensional collagen lattices (COL). (B) Cells were subcultured on tissue culture plates (TC) and in three-dimensional lattices (COL) for the time indicated. (C) Collagen gel mix was preincubated with anti-PDGF at 100 ng/ml (lanes 2 and 6) and at indicated concentrations (lanes 10 and 11), and with anticyclin A at 100 ng/ml (lane 12). Cells in collagen gel (lanes 5, 6, and 10–12) or on TC (lanes 3, 4, and 8) were stimulated with 30 ng/ml PDGF-BB preincubated with (lanes 4, 6, 10, and 11) or without anti-PDGF (lanes 3, 5, 8, and 9) or with anti-cyclin A (lane 12) 4 h after subculture. Total RNA was probed with human $\alpha_2$ integrin or MMP-1 cDNAs as indicated. Equal loading was confirmed by hybridization of the same blot with $[^{32}P]$ labeled probe for 28S ribosomal RNA. Results are representative of two experiments.

**Figure 2.** Inhibition of collagen gel induction of $\alpha_2$ and MMP-1 mRNA levels. (A and B) Cells were preincubated with inhibitors before subculture. (A) 10 $\mu$g/ml cycloheximide (CHX), a protein synthesis inhibitor, for 15 min. (B) PKC inhibitors bisindolylmaleimide GF 109203X (BIM) and calphostin C (CalC) at concentrations indicated for 1 h and 30 min, respectively. The incubation with inhibitors was continued for 18 h after subculture. (C) Quiescent cells either untreated or incubated for 24 h with PMA (300 ng/ml) were subcultured in collagen lattices or stimulated with PMA (50 ng/ml) for 16 h. Total RNA was probed with human $\alpha_2$ integrin and MMP-1 cDNAs. Equal loading was monitored by UV light examination of ethidium bromide–stained gel and confirmed by hybridization of the same blot with $[^{32}P]$ labeled probe for 28S ribosomal RNA. Results are representative of two independent experiments. T, tissue culture plates; C, collagen lattices.
expression (Fig. 2 B). Chelerythrine, however, did not demonstrate the inhibitory effect on the induction (data not shown).

Since there are 12 PKC isoforms identified so far, nine of which are phorbol 12-myristate 13-acetate (PMA)/DAG-inducible, whereas three were not induced by PMA, the identity of the PKC isoform(s) that mediated collagen-induced \( \alpha_2 \) and MMP-1 mRNA expression was sought. As a first step, we investigated whether this PKC was PMA-inducible or not. A widely used strategy is to deplete cellular PMA-inducible PKC levels by treating cell culture chronically with PMA (Larrodera et al., 1990). This approach depletes PKC isoforms sensitive to PMA/DAG activation. Thus, quiescent human fibroblast cultures were exposed to PMA (300 ng/ml) for 24 h before they were subcultured into collagen gel. PMA induction of cells cultured on tissue culture plates were performed in parallel as a control. \( \alpha_2 \) and MMP-1 mRNA steady-state levels were then determined. As expected, PMA was unable to induce either \( \alpha_2 \) or MMP-1 mRNA in cells chronically treated with PMA (Fig. 2 C, right lane). In contrast, collagen gel promoted a potent response in \( \alpha_2 \) mRNA expression in PMA-pretreated cells, which was only slightly lower than that in untreated cells (Fig. 2 C, middle two lanes). This result indicates the involvement of atypical PKC(s). However, MMP-1 mRNA response to collagen gel was reduced from 10-fold to 6.3-fold increase in cells chronically treated with PMA (Fig. 2 C, middle two lanes). These results suggest that collagen lattices activate an atypical PKC isoform that, to a different extent, is required for \( \alpha_2 \) and MMP-1 mRNA expression.

The Expression of Atypical PKC Isoforms

To determine which atypical PKC may be required for the collagen gel induction, protein levels of atypical PKC isoforms in human dermal fibroblasts were first examined. We performed Western analysis of total cell extracts after cells were grown in collagen gels for various time periods. In these experiments, HeLa cell extracts were used as a positive control (data not shown), as they contain all of the PKC isoforms examined (Chun and Jacobson, 1996). All three known members of the atypical PKC subfamily, \( \zeta \), \( \tau \), and \( \lambda \), were detected in human dermal fibroblasts (Fig. 3). The antibody against the PKC-\( \zeta \) isoform detected a high level of immunoreactive proteins from the lysates. Collagen gel did not change its amount. PKC-\( \lambda \) and -\( \tau \) were detected at relatively low levels in both monolayer and collagen gel cultures, and no obvious differences could be discerned in the proteins from the two culture conditions (Fig. 3). When lysate amount, antibody concentration, or exposure time was increased, the results became questionable because of the high background. A novel PKC isoform, PKC-\( \mu \), was also examined. Western blot detected PKC-\( \mu \) at a level comparable with PKC-\( \zeta \). In contrast to atypical PKC isoforms that were not synthetically affected by the collagen gel culturing, the cellular protein level of PKC-\( \mu \) decreased as cells were incubated in collagen gel for 18 h (Fig. 3). These results demonstrate that collagen lattices, which were capable of altering some PKC isoform protein levels, did not change the protein amount of atypical PKCs in human fibroblasts.

Collagen Gel Induction of PKC-\( \zeta \) Kinase Activity

Since collagen gel induction of \( \alpha_2 \) integrin and MMP-1 mRNA involved atypical PKC isoforms, collagen lattices may induce atypical PKC enzyme activity. The kinase activity of PKC-\( \zeta \) was investigated for the following reasons. PKC-\( \zeta \) is CalC (Larivee et al., 1994) and BIM sensitive (Xu et al., 1996), chelerythrine insensitive (Thompson and Fields, 1996), and not down-regulated by PMA (Wooten, 1994), which is consistent with the results observed thus far (Fig. 2, B and C). Furthermore, PKC-\( \zeta \) was the only member of atypical PKC subfamily detected in human fibroblasts in significant amounts (Fig. 3). To assess whether collagen gel can induce PKC-\( \zeta \) kinase activity, fibroblasts grown in collagen gel for various time periods were assayed for PKC-\( \zeta \) kinase activity. PKC-\( \zeta \) present in the cell extracts was immunoprecipitated with a polyclonal antibody. PKC-\( \zeta \) was autophosphorylated by kinase activities associated with the immunoprecipitates. The kinase activity was induced 30 min after fibroblasts cultured in collagen gel (4.6-fold), reached the maximum at 4 h (9.2-fold), and decreased after 4 h (Fig. 4, A and C). Cells grown on tissue culture plates, however, remained unstimulated during the entire time course (data not shown). The presence of phosphatidylserine, a PKC activator, increased

![Figure 3. Western analysis of PKC isoforms in total cell lysates. Normal human dermal fibroblasts were cultured 1 d in 1% FCS/DME before subculture in test conditions. Cells were subcultured on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) for the time indicated. Total cellular proteins were extracted, quantified with BCA assay, blotted, and detected with antibodies against PKC-\( \zeta \), -\( \tau \), -\( \lambda \), and -\( \mu \).](Image 308x444 to 548x750)
Collagen Gel Induction of DNA Binding Activity of Transcription Factor NF-κB

PKC-ζ has been reported as a positive regulator of the activity of a transcription factor, NF-κB (Diaz-Meco et al., 1993). Cells that display enhanced PKC-ζ phosphorylation activity also increase NF-κB DNA binding activity in response to sphingomyelinase (Lozano et al., 1994), ras p21 (Diaz-Meco et al., 1993), and TNF-α (Muller et al., 1995). The induction of PKC-ζ activity by cells incubated in collagen gels prompted us to ask whether NF-κB DNA binding activity was also induced. Nuclear extracts were prepared from human fibroblasts grown in collagen gel from 30 min to 24 h. The NF-κB DNA binding activity present in the nuclear extracts was detected by gel mobility shift assay using a DNA probe encompassing the κB motif (see Materials and Methods). As seen in Fig. 4 B, a 30-min incubation of human fibroblasts in collagen gel induced the formation of specific κB DNA–protein complexes. The NF-κB DNA binding activity remained similar from 30 min to 4 h (Fig. 4 C, 2.9–3.8-fold increases) but increased modestly after 24 h of incubation in collagen gel (Fig. 4 C, 4.9-fold increases). The quantification of the NF-κB DNA binding activity revealed a kinetic pattern in concord with, if not identical to, that of PKC-ζ kinase activity (Fig. 4 C). Cells grown on tissue culture plates, on the contrary, did not demonstrate any change in the NF-κB DNA binding activity (data not shown). Competition experiments with an unlabeled κB consensus sequence confirmed the specificity of the binding complexes. As a control, the binding activity to the Sp1, a transcription factor, consensus sequence was also examined (Fig. 4 B). The Sp1 DNA binding activity present in nuclear extracts was not altered by cells cultured in collagen gel. Therefore, we conclude that this three-dimensional cell culture system increased PKC-ζ activity in association with the activation of NF-κB DNA binding complexes. The composition of the NF-κB DNA binding complexes was examined next. The supershift assay showed that p50 of NF-κB/Rel family proteins was one component of the binding complex (Fig. 4 D, II) regardless of culture condition. Antibody to p65, however, only shifted a very low amount of the binding complex (Fig. 4 D, I), suggesting that the binding complex may be composed of other member(s) of NF-κB/Rel family. Therefore, collagen gel induced the p50-containing complex binding to NF-κB site without changing protein composition.

Antisense Translational Inhibition of PKC-ζ

The results presented so far have suggested that collagen gel was an activator of PKC-ζ activity and that collagen gel–induced α2 and MMP-1 mRNA expression required atypical PKC isoform activity. The potential role of PKC-ζ in collagen gel–induced α2 and MMP-1 mRNA expression was then investigated. To establish a direct connection between these two events, we used a previously described strategy to remove PKC-ζ protein through translation inhibition by antisense oligonucleotides (Xu et al., 1996). Antisense phosphorothioate oligonucleotides 1142 and 1144 have sequences targeted at the beginning of the open reading frame of PKC-ζ cDNA (Barbee et al., 1993). This GC-rich site is in a nonconserved variable region (V1) of PKC family (Nishizuka, 1992), which differs significantly among

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**Figure 4.** Stimulation of PKC-ζ and NF-κB DNA binding activity by three-dimensional collagen lattices. Normal human dermal fibroblasts were cultured 1 d in 1% FCS/DME before subculture in test conditions. Cells were subcultured on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) for the times indicated. (A) PKC-ζ kinase activity assay. Total cellular proteins were extracted, quantified with BCA assay, and immunoprecipitated with a polyclonal antibody against PKC-ζ in the presence or absence of a synthetic peptide to which the antibody was raised (Peptide). The immunoprecipitates were incubated in a kinase assay buffer for 10 min at 30°C. Unless specified, the reactions were carried out in the absence of phosphatidyserine. The kinase activity was determined by autophosphorylation as described in Materials and Methods. The results are representative of three independent experiments. (B) Gel mobility shift assay. Nuclear extracts were prepared and assayed for DNA binding activity of NF-κB and Sp1 by gel mobility shift as described in Materials and Methods. Arrows indicate specific bindings. The results are representative of three independent experiments. (C) Quantification of A and B. (D) Supershift assay. Nuclear extracts prepared from cells stimulated with collagen gel (COL) for 24 h and on tissue culture plates (TC) were incubated with labeled NF-κB consensus sequences before further incubation with antibodies against p65 and p50. I, the supershift caused by p65; II, the supershift caused by p50; III, the specific NF-κB binding. The results of these experiments are consistent with the hypothesis that collagen gel–induced production of the PKC-ζ activity impressively from unstimulated (seven-fold) and slightly from stimulated cells (11.5-fold, a 20% increase). Therefore, collagen gel appears to be an activator for fibroblast PKC-ζ, as determined by kinase activity associated with PKC-ζ immunoprecipitates.
PKC isoforms. Antisense oligonucleotides 1142 and 1144 are essentially the same except that 1144 was designed to have an additional looping secondary structure at its 3’ end to reduce the possible degradation caused by 3’ exo-nuclease (Tang et al., 1993). Previously we have found that the antisense oligonucleotides at concentrations higher than 2.5 μM can inhibit human fibroblast PKC-ζ protein level by at least 70% (Xu et al., 1996). Therefore, fibroblasts were lipid-transfected with antisense oligonucleotides 1142 or 1144 at 2.5 μM. As a control, some cells were transfected with “sense” oligonucleotide 1143. The transfected cells were then subcultured into collagen gels and further treated with oligonucleotides for 18 h. The depletion of PKC-ζ protein levels by antisense inhibition of translation was monitored by Western blotting (Fig. 5 A) and kinase activity of PKC-ζ immunoprecipitates (Fig. 5 B). The results from Fig. 5 demonstrated a significant reduction of PKC-ζ protein and its associated kinase activity after treatment with both oligonucleotides, 1142 and 1144. The specificity of antisense inhibition was confirmed by probing the membrane with an mAb against PKC-α. The equal loading was confirmed by detection with an mAb against β-tubulin (Fig. 5 A).

Such cells were harvested for analysis of α2 and MMP-1 mRNA expression. Antisense treatment inhibited collagen gel induction of α2 expression up to 60% (Fig. 6). Both oligonucleotides 1142 and 1144 had similar effects. These results are consistent with observations of cells treated with the PKC inhibitors, BIM and CaCl2 (Fig. 2 B), and chronically with PMA (Fig. 2 C). MMP-1 expression was also inhibited by antisense treatment, although less effectively with 1142 and more with 1144. The expression of a fibronectin receptor integrin subunit, α5, was not affected by either a collagen gel environment or antisense treat-

Figure 5. Specific down-regulation of protein levels and PKC-ζ activity by antisense inhibition of translation. Subconfluent normal dermal human fibroblasts were either untreated or treated with sense (1143) or antisense oligonucleotides (1142 and 1144) at 2.5 μM in DME containing 20 μg/ml DOTMA for 6–8 h. After this time, the medium containing DOTMA was replaced by fresh medium containing appropriate oligonucleotides. Cells were subcultured 48 h later on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) and continuously incubated in appropriate oligonucleotides for 18 h. Total cellular proteins were extracted, quantified with BCA assay, and assayed for PKC-ζ in vitro kinase activity or protein level. (A) Western blot detection of PKC-ζ, PKC-α, and β-tubulin. (B) In vitro kinase activity in immunoprecipitates of PKC-ζ.

Figure 6. Antisense-mediated down-regulation of PKC-ζ protein inhibits collagen gel induction of integrin α2 and MMP-1 mRNA expression. Total cellular RNA was extracted from normal human dermal fibroblasts after incubation in the presence or absence of sense (1143) or antisense (1142 and 1144) oligonucleotides complementary to the 5’-end of the PKC-ζ transcript at 2.5 μM. Before RNA harvest, cultures were incubated in collagen lattices for 18–24 h. Total RNA was probed with human α2 integrin, MMP-1 and α5 integrin cDNAs. Equal loading was monitored by UV light examination of ethidium bromide–stained gel and confirmed by hybridization of the same blot with [32P]labeled probe for 28S ribosomal RNA. Results are representative of two independent experiments.
ment, confirming the specificity of the antisense inhibition. Therefore, PKC-ζ is involved in collagen gel induction of the α2 integrin subunit and MMP-1 mRNA expression.

The Cytoplasmic and Nuclear Localization of PKC-ζ

Several PKC isoforms have been reported to be present in the nucleus, including PKC-δ and PKC-ε (Ventura et al., 1995). The nuclear localization of those PKC isoforms seems directly correlated to its functional role in regulating cellular biosynthetic activities. For example, the phorbol ester-regulated expression of opioid peptide gene in rat myocardial cells was reported as a possible target of nuclear PKC-ζ and -ε through autocrine or paracrine mechanisms (Ventura et al., 1995). The involvement of PKC-ζ in mRNA expression of α2 and MMP-1 prompted us to ask whether PKC-ζ is a cytosolic or nuclear protein in human dermal fibroblasts. Previously, PKC-ζ has been reported present in cytoplasm and nucleus in species such as rat and rabbit (Masmoudi et al., 1989; Hagiwara et al., 1990; Disatnik et al., 1994; Rosenberger et al., 1995). To assess this possibility of human PKC-ζ, the distribution of PKC-ζ as well as -μ in cytoplasm and nucleus was examined. Intact nuclei were isolated from fibroblasts grown as monolayer or in three-dimensional collagen gel. Western analysis was performed with cytoplasmic and nuclear fractions. While PKC-μ was predominantly present in the cytoplasm, PKC-ζ was detected in both cytoplasm and nucleus (Fig. 7). Cells grown in collagen gel did not change the distribution between the two subcellular compartments. Therefore, PKC-ζ, the kinase required for collagen gel regulatory pathway leading to α2 and MMP-1 mRNA expression in human dermal fibroblasts, is constitutively a nuclear as well as a cytoplasmic protein.

Discussion

We have previously shown that a three-dimensional collagen lattice, either stressed or relaxed, can induce integrin α2 mRNA expression in human dermal fibroblasts (Xu and Clark, 1996). Interestingly, no induction of α2 or MMP-1 mRNA is observed when fibroblasts are plated on a collagen monolayer (Fig. 1 A) as previously reported by Langholz et al. (1995) for MMP-1. The evidence presented in this report indicates that PKC-ζ is required for the full response of integrin α2 subunit and MMP-1 mRNA to collagen lattice (Fig. 6).

Although the nature of precise primary signals triggered by a three-dimensional type I collagen construct is undefined, we report here that these collagen gels activate PKC-ζ (Fig. 4 A) as a component of secondary transduction signals (second messenger pathway). How might collagen gel activate PKC-ζ? Previously, we showed that PDGF can induce PKC-ζ activity (Xu et al., 1996). Could collagen gel use the same pathway as PDGF to activate PKC-ζ? In fact, adhesion molecules such as CAMs (cell adhesion molecules) and basic fibroblast growth factor both activate FGF receptor to induce cell contact-dependent neurite outgrowth (Williams et al., 1994). However, several lines of evidence argue against the similar scheme with collagen gel and PDGF. First, Lin and Grinnell (1993) have presented evidence that PDGF, but not collagen gel, induces tyrosine phosphorylation of PDGF receptor in fibroblasts. Second, the same report also showed that collagen gel, especially relaxed collagen gel, actually reduces PDGF-stimulated receptor autophosphorylation. Third, we previously reported that collagen gel does not stimulate expression of integrin α2 and α3 subunits, two PDGF-inducible genes (Xu and Clark, 1996). Fourth, collagen gel possesses both positive and negative impact on PDGF stimulation of integrin subunit mRNA expression: positive on α2, negative on α3 and α5 (Xu and Clark, 1996). The data taken together, in fact, indicate that collagen gel interferes with some PDGF pathways. Thus, collagen gel and PDGF signaling pathways appear not to converge at PDGF receptor site.

Among known activators of PKC-ζ are AA generated by phospholipase A2 and ceramide generated by phosphatidylycholine-hydrolyzing phospholipase C (PC-PLC)/sphingomyelinase. Adhesion of HeLa cells to collagen (Chun and Jacobson, 1993) or β1 integrin clustering (Auer and Jacobson, 1995) increased the release of AA. AA could activate PKC-ζ either directly (Nakanishi and Exton, 1992) or indirectly by triggering DAG release (Auer and Jacobson, 1995). DAG released from AA metabolism may activate PKC-ζ by sphingomyelinase-ceramide pathway since it was able to increase sphigomyelin hydrolysis in some cell types (Kolesnick, 1987). Alternatively, collagen gel may sequentially activate PC-PLC and PKC-ζ. In our system, disruption of PC-PLC activity with its inhibitor D609 (Schutze et al., 1992) blocked collagen gel induction of α2 mRNA expression (data not shown), implicating the importance of this pathway. Another possibility for PKC-ζ activation by collagen gel may be its phosphorylation by a second serine/threonine kinase. Several reports have suggested that the phosphorylation may precede the PKC activation (Pears et al., 1992; Cazaubon and Parker, 1993). This phosphorylation requirement has been shown with PKC-α and -β (Cazaubon et al., 1994; Orr and Newton, 1994). Potential phosphorylation sites are thought to be present in several members of PKC family, including ζ (Tsutakawa et al., 1995). In agreement with this, the clustering of α2 and β1, the subunits of collagen receptor integrin α2β1, which was reported to mediate collagen gel-induced

Figure 7. Western analysis of PKC isoforms in cytoplasm and nuclei. Normal human dermal fibroblasts were starved 1 d in 1% FCS/DME before subculture in test conditions. Cells were subcultured on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) for 18–24 h. Nuclear (Nuclei) and cytoplasmic (Cell Extracts) fractions were prepared, quantified with BCA assay, blotted, and detected with antibodies against PKC-ζ and -μ. Figure 7.
MMP-1 expression (Langholz et al., 1995), can induce p21ras activation (Kapron-Bras et al., 1993). Ras has been shown to be coprecipitated with PKC-ζ and to induce PKC-ζ activity (Diaz-Meco et al., 1994b). Indeed, our data showed that collagen gel did not alter the cellular level of PKC-ζ (Fig. 3). Thus, posttranslational modification is a possible mechanism by which PKC-ζ is regulated.

The downstream targets of PKC-ζ activity are yet to be discovered. One consequence of PKC-ζ activation by extracellular stimuli such as TNF-α is the induction of NF-κB activity (Diaz-Meco et al., 1994a). Here we present evidence from gel mobility shift assays that collagen lattices induced NF-κB DNA binding activity in dermal fibroblasts rapidly (≤30 min) and persistently (≥24 h) (Fig. 4 B). This is in concordance with the observation that cells stimulated with TNF-α and p21Ras demonstrate both increased PKC-ζ and NF-κB activities (Diaz-Meco et al., 1994a; Muller et al., 1995). In fact, TNF-α was also shown to induce NF-κB DNA binding rapidly and persistently (1/3–20 h) (Johnson et al., 1996; Roff et al., 1996), similar to collagen gel induction. One puzzling observation was that during a 24-h incubation period, PKC-ζ kinase activity peaked at 4 h, whereas NF-κB DNA binding reached the maximum at 24 h with modest increase over the binding at 30 min (Fig. 4 C). While rapid increase of NF-κB activity is predominantly caused by posttranslational modification (for review see Siebenlist et al., 1994), sustained nuclear NF-κB activity over longer periods of stimulation may be caused by either sustained reduction of NF-κB inhibitor B, as demonstrated in vascular endothelial cells (Johnson et al., 1996), or induced expression of NF-κB subunits other than RelA (p65), as reported for HL60 cells (Hohmann et al., 1991). Both c-Rel and RelB can be transcriptionally regulated through the κB element in their promoters (Hannink and Temin, 1990; Ryseck et al., 1992), which p65 lacks (Ueberla et al., 1993). Stimulation of Jurkat cells over several hours results in increasing amounts of c-Rel relative to p65 in the nucleus, possibly because of preferentially induced levels of c-Rel (Molitor et al., 1990; Doerre et al., 1993). In support of this, results from supershift assay showed very low levels of p65 in the κB-binding complex (Fig. 4 B). We speculate that the rapid binding observed may be caused by phosphorylation of existing NF-κB/Rel in cellular pool, whereas the persistent binding may result from the newly synthesized NF-κB/Rel. Therefore, although PKC-ζ reached the maximum at 4 h, NF-κB binding activity may not show identical kinetics and fold-increases because of regulatory transition from posttranscriptional modification of existing protein factors to synthesis and modification of new proteins. In agreement with this, we obtained evidence that collagen gel induction of α2 and MMP-1 mRNA expression, a PKC-ζ-mediated event, requires both NF-κB (unpublished data) and protein synthesis (Fig. 2 A).

It is of great interest that collagen gel and PDGF (Xu et al., 1996) both require PKC-ζ activity to induce integrin α2 mRNA expression. How is the induction further enhanced when both signals are present (Fig. 1 C)? Although the study is at a stage too early to provide answers, there are several possibilities. First, PKC-ζ activity has been shown to be regulated by protein–protein interactions with apoptosis gene par-4 product (Diaz-Meco et al., 1996b), λ-interacting protein (LIP) (Diaz-Meco et al., 1996a), and Ras (Diaz-Meco et al., 1994b). Thus, collagen gel and PDGF might induce different protein factors that in turn activate PKC-ζ by protein–protein interaction. Copresence of both stimuli could synergistically induce the kinase activity by the interaction among multifactors. Second, there are multiple potential phosphorylation sites in PKC-ζ molecules (Tsutakawa et al., 1995). The fact that PKC-ζ is physically interacting with and activated by Ras (Diaz-Meco et al., 1994b) suggests that phosphorylation is an important means of regulating its activity. Thus, collagen gel and PDGF may activate PKC-ζ by inducing phosphorylation at different amino acid residue(s). A similar scheme was suggested in the synergistic activation of NF-κB by calcium and Ras/Raf (for review see Baeuerle and Baltimore, 1996). Third, PKC-ζ can induce transactivating activities of different transcription factors such as NF-κB and AP-1 (Bjorkoy et al., 1995). A differentially activated PKC-ζ could stimulate distinct downstream pathways. We have observed that collagen gel stimulated DNA binding activity of NF-κB (Fig. 4 B) but not AP-1 (unpublished data). In contrast, PDGF induced DNA binding of substantial AP-1 but little NF-κB (unpublished data). It is tempting to suggest that collagen gel/PKC-ζ and PDGF/PKC-ζ activate different transcription factors to induce integrin α2 and MMP-1 mRNA expression. The synergistic action then would occur at the level of gene transcription. Investigations of these possibilities are ongoing in our laboratory.

It is well established that three-dimensional collagen matrix sends integrated physical and chemical signals to the interior of the cell. Nevertheless, detailed knowledge of collagen matrix signal transduction is very limited. Results reported here connect a signal transduction protein, PKC-ζ, to the upstream initiator, the collagen lattice, and to downstream outputs, α2 and MMP-1 mRNA expression.

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References

Ahlen, K., and K. Rubin. 1994. Platelet-derived growth factor-BB stimulates synthesis of the integrin α5 subunit in human diploid fibroblasts. Exp. Cell Res. 215:347–353.

Auer, K.L., and B.S. Jacobson. 1995. β1 integrins signal lipid second messengers required during cell adhesion. Mol. Biol. Cell 6:1305–1313.

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

Barbee, J.L., S.L. Deutscher, C.R. Loomis, and D.J. Burns. 1993. The cDNA sequence encoding human kinase C-ζ. Gene. 132:305–306.

Bjorkoy, G., A. Overvatn, M.T. Diaz-Meco, J. Moscat, and T. Joensuu. 1995. Evidence for a bifurcation of mitogenic signaling pathway activated by Ras and phosphatidylycholine-hydrolyzing phospholipase C. J. Biol. Chem. 270:21299–21306.

Cazaubon, S.M., and P.J. Parker. 1993. Identification of the phosphorylated region responsible for the permissive activation of protein kinase C. J. Biol. Chem. 268:17559–17563.

Cazaubon, S., F. Bornancin, and P.J. Parker. 1994. Threonine-497 is a critical site for permissive activation of protein kinase Ca. Biochem. J. 301:443–448.

Chromczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Bio-
Klein, C.E., D. Dressel, T. Steinmayer, C. Mauch, B. Eckes, T. Krieg, R.B. Johnson, D.R., I. Douglas, A. Jahnke, S. Ghosh, and J.S. Pober. 1996. A sus-

Hagiwara, M., C. Uchida, N. Usuda, T. Nagata, and H. Hidaka. 1990.

Diaz-Meco, M.T., J. Lozano, M.M. Municio, E. Berra, R.T. Hay, T.W. Sturgill, and J. Moscat. 1994. PKC induces pho-

sphorylation and inactivation of IκBα in vitro. EMBO (Eur. Mol. Biol. Or-

gan. J.) 13:2842–2848.

Diaz-Meco, M.T., J. Lozano, M.M. Municio, E. Berra, S. Frutos, L. Sanz, and J. Moscat. 1994. Evidence for the in vivo and in vitro interaction of ras with protein kinase C. J. Biol. Chem. 269:3706–3710.

Diaz-Meco, M.T., M.M. Municio, P. Sanchez, and J. Lozano. 1994a. X-Interact-
ing protein, a novel protein that specifically interacts with the zinc finger do-

main of the atypical protein kinase C isozyme λ and stimulates its kinase ac-

tivity in vitro and in vivo. Mol. Cell. Biol. 16:105–114.

Diaz-Meco, M.T., M.M. Municio, S. Frutos, P. Sanchez, J. Lozano, L. Sanz, and J. Moscat. 1996b. The product of par-4, a gene induced during apoptosis, in-
teracts selectively with the atypical isoforms of protein kinase C. Cell. 86:

767–786.

Disatnik, M.H., G. Buraggi, and D. Mochly-Rosen. 1994. Localization of pro-

tein kinase C isoforms in cardiac myocytes. Exp. Cell. Res. 210:280–287.

Doerre, S., P. Sista, S.-C. Sun, D.W. Ballard, and W.C. Greene. 1993. The c-er-

protooncogene product represses NF-κB p65-mediated transcriptional acti-

vation of the hypoxiainducible factor-1. Proc. Natl. Acad. Sci. USA. 90:1023–1027.

Eckes, B., C. Mauch, G. Huppe, and T. Krieg. 1993. Downregulation of col-

lagen synthesis in fibroblasts within three-dimensional collagen lattices in-
volves transcriptional and posttranscriptional mechanisms. FEMS Lett. 318:

129–133.

Ferns, G.A.A., E.W. Raines, K.H. Sprugel, A.S. Motani, M.A. Reidy, and R.

Ross. 1991. Inhibition of neontinal smooth muscle accumulation after an-

gioplasty by an antibody to PDGF. Science (Wash. DC). 253:1129–1132.

Gimond, C., A. de Melker, M. Aumailler, and A. Sonnenberg. 1995. The cyto-

plastic domain of αβ6 integrin subunit is an in vitro substrate for virus-

protein kinase C. Cell. 84:232–235.

Greenberg, M.E., and F.B. Ziff. 1984. Stimulation of 3T3 cells induces tran-
scription of the c-fos proto-oncogene. Nature (Lond.). 311:433–438.

Gniffter, F. 1994. Fibroblasts, myofibroblasts, and wound contraction. J. Cell

Biol. 125:401–404.

Hagiwara, M., C. Uchiha, N. Usuda, T. Nagata, and H. Hidaka. 1990a. Relative protein kinase C in nuclei of nerve cells. Biochem. Biophys. Res. Commun. 168:161–168.

Hamann, M., and H.M. Temin. 1990. Structure and autoregulation of the c-erl promoter. Oncogene. 5:1843–1850.

Hay, E.D. 1991. Collagen and other matrix glycoproteins in embryogenesis. Cell Biology of the Extracellular Matrix. E.D. Hay, editor. Plenum Press, New York. 419–462.

Hohmann, H.-P., R. Reny, C. Scheideret, and A.P.G.M. vanLoon. 1991. Main-
tenance of NF-κB activity is dependent on protein synthesis and the continu-
ous presence of external stimuli. Mol. Cell. Biol. 11:259–266.

Johanns, D.R., I. Douglas, A. Jahnke, S. Ghosh, and J.S. Pober. 1996. A sus-
tained reduction in IκB-β may contribute to persistent NF-κB activity in human endothelial cells. J. Biol. Chem. 271:16317–16322.

Kapron-Bras, C., L. Fitz-Gibbon, P. Jeevanantham, J. Wilkins, and S. Dedhar. 1993. Stimulation of tyrosine phosphorylation and accumulation of GTP-

bound p21ras upon antibody-mediated α2β1 integrin activation in T-lym-

phocytic cells. J. Biol. Chem. 268:20701–20704.

Klein, C.E., D. Dressel, T. Steinnmayer, C. Mauch, E. Tkeik, R.B. Bifulco, and J.S. Pober. 1991. Integrin αvβ3 is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. J. Cell. Biol. 115:1427–1436.

Kolesnick, R.N. 1987. 1,2-Diacylglycerols but not phorbol ester stimulate sphin-
gomyelin hydrolysis in GH3 pituitary cells. J. Biol. Chem. 262:16769–16772.

Langholz, O., D. Rockel, C. Mauch, E. Kozloski, I. Bank, and T. Krieg. 1995. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by α1pI and α2pI integrins. J. Cell Biol. 131:1903–1915.

Larivée, P., S.J. Levine, A. Martinez, T. Wu, C. Logun, and J.H. Shelhamer. 1994. Inducer-activating role of C/EBP in the transactivation of protein kinase C expression. Proc. Natl. Acad. Sci. USA. 91:10143–10147.

Lin, C.-Q., and M.J. Bissell. 1993. Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J. 7:737–743.

Liu, Y., and F. Grinnell. 1995. NCAM; a candidate cell surface marker of epi-

defferentiated epithelial cells. J. Cell. Biol. 124:679–682.

Liu, Z. and B. Yao. 1993. Role of NF-κB in the growth and invasion of mam-

mary carcinoma cells. J. Cell. Biol. 127:1295–1303.

Liu, Z., and B. Yao. 1993. The role of NF-κB in the growth and invasion of mam-

mary carcinoma cells. J. Cell. Biol. 127:1295–1303.

Liu, Z., and B. Yao. 1993. The role of NF-κB in the growth and invasion of mam-

mary carcinoma cells. J. Cell. Biol. 127:1295–1303.

Liu, Z., and B. Yao. 1993. The role of NF-κB in the growth and invasion of mam-

mary carcinoma cells. J. Cell. Biol. 127:1295–1303.

Liu, Z., and B. Yao. 1993. The role of NF-κB in the growth and invasion of mam-

mary carcinoma cells. J. Cell. Biol. 127:1295–1303.
moter. *J. AIDS Res.* 6:227–230.

Unemori, E.N., and Z. Werb. 1986. Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. *J. Cell Biol.* 103:1021–1031.

Ventura, C., G. Pintus, I. Vaona, F. Bennardini, G. Pinna, and B. Tadolini. 1995. Phorbol ester regulation of opioid peptide gene expression in myocardial cells: role of nuclear protein kinase C. *J. Biol. Chem.* 270:30115–30120.

Vuori, K., and E. Ruoslahti. 1993. Activation of protein kinase C precedes $\alpha_5\beta_1$ integrin-mediated cell spreading on fibronectin. *J. Biol. Chem.* 268:21459–21462.

Williams, E.J., J. Furness, F.S. Walsh, and P. Doherty. 1994. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron.* 13:583–594.

Woods, A., and J.R. Couchman. 1992. Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* 101:277–290.

Wooten, M.W., G. Zhou, M.L. Seibenhener, and E.S. Coleman. 1994. A role for $\zeta$ protein kinase C in nerve growth factor-induced differentiation of PC12 cells. *Cell Growth Differ.* 5:395–403.

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

Xu, J., M.M. Zutter, S.A. Santoro, and R.A.F. Clark. 1996. PDGF induction of $\alpha_2$ integrin gene expression is mediated by protein kinase C$\zeta$. *J. Cell Biol.* 134:1301–1311.