PDGF Induction of α_2 Integrin Gene Expression Is Mediated by Protein Kinase C-ζ

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Abstract. Platelet-derived growth factor (PDGF) stimulates fibroblasts to move over collagen and contract three-dimensional collagen gels, processes important in wound repair and fibrocontractive diseases. These processes depend on $\alpha_2\beta_1$ integrin ligation of collagen and PDGF induces the expression of this integrin. Several lines of evidence presented here suggest that PKC-ζ plays a role in α_2 integrin gene expression. The induction was blocked by chemical inhibitors for protein tyrosine kinases (PTK), genistein, and protein kinase C (PKC), chelerythrine, and bisindolylmaleimide GF 109203X. Cells depleted of phorbol 12-myristate 13-acetate (PMA)-inducible PKCs by chronic treatment with PMA still demonstrated an α_2 response to PDGF indicating that a non–PMA-sensitive PKC isoform was required. PDGF induced kinase activity in PKC-ζ immunoprecipitates. Antisense oligonucleotides complementary to 5′ end of PKC-ζ mRNA sequences blocked the PDGF-induced increase of α_2 mRNA levels up to 70%, indicating PKC-ζ, a non–PMA-sensitive PKC isoform, is a component of the PDGF stimulatory pathway for α_2 mRNA synthesis. A 961-base pair (bp) upstream region of α_2 gene/CAT construct transfected into human dermal fibroblasts was positively regulated by PDGF as judged by CAT enzymatic levels. Both PTK and PKC inhibitors blocked PDGF-stimulation of the α_2 promoter fragment/CAT construct, indicating that the phosphorylation requirement occurred at α_2 promoter-directed transcription level. Therefore, we propose that PDGF-stimulatory pathway of α_2 integrin gene expression involves multiple cellular protein kinases, one of which is PKC-ζ.

CELL adhesion and migration are important elements in such physiological processes as wound healing, inflammation, differentiation and development, and in the pathobiology of tumor invasion. The integrin superfamily plays a major role in the mediation of adhesive interaction between cells and extracellular matrices. Integrins are heterodimers composed of an α chain and a β chain. β1 integrin can form a complex with at least ten different α subunits, namely α_1–α_9 and α_v (Haas and Plow, 1994; Hynes, 1992). Among them, $\alpha_2\beta_1$ integrin is known to mediate cell adhesion to and migration on type I collagen (for review see Santoro and Zutter, 1995 and references therein). In the late phase of wound healing, tissue contraction also probably requires the $\alpha_2\beta_1$ integrin based on previous in vitro experiments (Schiro et al., 1991). The cellular level of integrins in part determines the adhesive behavior of cells and is subject to modulation by growth and differentiation factors. The regulatory stimuli for α_2 integrin subunit expression include platelet-derived growth factor (PDGF)[1] (Ahlen and Rubins, 1994; Xu and Clark, 1996), transforming growth factor-β (Riikonen et al., 1995), epidermal growth factor (Fujii et al., 1995), and its ligand, collagen (Klein et al., 1991; Xu and Clark, 1996).

PDGF elicits a wide range of physiological responses such as development, wound healing, inflammation, and oncogenesis. The mitogenic effect of PDGF is mediated through receptors that possess an intrinsic tyrosine kinase activity (for review see Claesson-Welsh, 1994). Binding of PDGF to its cell surface receptor causes receptor dimerization and phosphorylation of substrates including the receptor itself and other intracellular proteins that associate with the tyrosine-phosphorylated receptors. Those proteins include enzymes that generate active second messenger molecules such as phospholipase Cγ, phosphatidylinositol 3′-kinase, protein kinases (Src and Raf) and phosphatases (PTP-1D), and adaptor proteins such as Shc that are thought to link activated receptors to the Ras signaling pathway. The biochemical properties of these effec-

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1. *Abbreviations used in this paper*: DAG, diacylglycerol; MAPK, mitogenic-activated protein kinase; NF, nuclear factor; PDGF, platelet-derived growth factor; PTK, protein tyrosine kinase; PKC, protein kinase C; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

tor proteins are believed to be modified as a consequence of association with and phosphorylation by the receptor. Thus, PDGF triggers a diverse array of downstream early signaling events.

One cellular response to PDGF stimulation is gene induction. PDGF responsive genes include immediate early genes such as *egr-1* (Mundschau et al., 1994), *c-fos* (Greenberg and Ziff, 1984), and *c-jun* (Rauscher et al., 1988), slow immediate genes such as c-*myc* (Kelly et al., 1983) and chemokine gene *MCP-1 (JE/MCP-1)* (Freter et al., 1995), and late genes such as stromelysin (Diaz-Meco et al., 1991) and interstitial collagenase (Circolo et al., 1991). However, the events linking early second messenger activation by PDGF with subsequent gene expression are unclear.

One such candidate is protein kinase C (PKC). A family of serine/threonine-specific protein kinases, PKC has been linked to cell proliferation, differentiation, and regulation of gene expression. This enzyme family can be divided into three groups (Nishizuka, 1995). The classic group containing isoforms α, βI, βII, and γ depends on Ca^{2+} and phorbol ester/diacylglycerol (DAG) for activity. The nonclassic group containing isoforms δ, ϵ, η, Θ, and μ is phorbol ester/DAG-dependent but does not require Ca^{2+}. An atypical group containing PKC-λ, τ, and ζ is not activated by phorbol ester/DAG. PKC has been associated with various PDGF-stimulated cellular activities such as Na^+/H^+ exchange in normal murine mammary gland epithelial (NMuMG) and Chinese hamster ovary (CHO) cells (Ma et al., 1994) and C3H 10T1/2 (Schwartz and Lechene, 1992); formation of prostaglandins in NIH 3T3 fibroblasts (Finkenzeller et al., 1993); proliferation of human mesangial cells (Choudhury et al., 1993), proliferation of vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) (Inui et al., 1994); translocation of 80-kD MARCKS in Swiss 3T3 cells (Herget and Rozengurt, 1994); downregulation of a major PKC substrate 80-kD/MARCKS in Swiss 3T3 fibroblasts (Brooks et al., 1992); transcription of vascular endothelial growth factor gene (VEGF) in NIH 3T3 fibroblasts (Finkenzeller et al., 1992); and slow immediate genes JE/MCP-1 (Freter et al., 1995) and late gene stromelysin (Sanz et al., 1994) in NIH 3T3 fibroblasts.

The mechanism by which PDGF regulates α_2 integrin gene expression has become a focus of our research. In this report, chemical inhibitors were used to investigate the involvement of both protein tyrosine kinases and protein kinase C in PDGF regulation of α_2 integrin gene expression. Furthermore, human dermal fibroblasts were transfected with α_2 promoter region/CAT constructs to establish the link between PDGF-induced kinase activity and α_2 promoter-directed gene transcription. Data from the studies indicated the involvement of an atypical PKC. Therefore, antisense oligonucleotides were used to determine that PKC-ζ activation was required for PDGF stimulation of α_2 gene expression.

Materials and Methods

Cell Culture and Reagents

Human dermal fibroblast cultures were established by outgrowth from healthy human skin biopsies (kindly provided by Marcia Simon, Department of Dermatology, SUNY at Stony Brook). The cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD), supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 U/ml streptomycin (GIBCO BRL), and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells between population doubling levels (PDL) 15 and 20 (the 6th and 10th passage) were used for the experiments. In experiments where inhibitors were used, levels of lactate dehydrogenase activity measured (Sigma LD Diagnostic kit) were similar regardless of cells that were cultured in the presence or absence of inhibitors. Plasmids p$\alpha_2$961-CAT and cytomegalovirus-CAT (CMV-CAT) have been described previously (Zutter et al., 1994). Plasmid RSV-β-galactosidase was kindly provided by Lorne Taichman, Department of Oral Biology and Pathology (SUNY at Stony Brook). PDGF-BB was generously provided by Charles Hart of ZymoGenetics (Seattle, WA). Human α_2 and α_3 cDNA probes were generously provided by Yoshikazu Takada of The Scripps Institute (San Diego, CA) (Takada and Hemler, 1989; Takada et al., 1991). α_5 cDNA was purchased from GIBCO BRL. Genistein was obtained from Sigma Chem. Co. (St. Louis, MO). Bisindolylmaleimide GF 109203X was purchased from CalBiochem (La Jolla, CA). Chelerythrine was purchased from LC laboratories (Woburn, MA). Polyclonal antibodies against human protein kinase C-ζ and -μ were purchased from GIBCO BRL and Santa Cruz Biotechnology (CA), respectively. Monoclonal antibody against β-tubulin was purchased from Chemicon (Temecula, CA). Phosphatidylserine was purchased from Sigma Chem Co. [^{14}C]Chloramphenical, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from Du Pont New England Nuclear (Boston, MA).

RNA Blot Analysis of Total Cellular RNA

Total RNA was isolated using a guanidinium thiocyanate method (Chromczynski and Sacchi, 1987). For Northern blot hybridization, 3–5 μg of total RNA was treated with glyoxal/DMSO, separated by electrophoresis on a 1% agarose gel in 10 mM phosphate buffer, pH 7.0, and transferred to Hybond$^+$ nylon membranes (Amersham Corp., Arlington Heights, IL). Ethidium bromide (0.5 μg/ml) was included in the gel to monitor equal loading by the quantity of 18S and 28S ribosomal RNA present. cDNA probes were labeled with [α-^{32}P]dCTP by the random primer procedure (Du Pont New England Nuclear). Oligonucleotide probes were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). The filters were hybridized to the labeled probes in QuickHyb solution (Stratagene, La Jolla, CA) for 3 h at 68°C and washed according to manufacturer's protocol. After autoradiography (Kodak X-Omat AR) at −80°C for optimal exposure, signal intensity was determined by densitometry. Values shown are representative of at least two independent experiments. An oligonucleotide complementary to 28S ribosomal RNA was used as an additional control of equal loading (Clontech, Palo Alto, CA).

DNA Transfection

Adult human dermal fibroblasts were passaged at 5–7 × 10^5 per 10-cm plate. Transfection was performed 22–24 h after passage. Plasmid DNA (15 μg per plate) and pRSV-βgal control DNA (15 μg per plate) in 1 ml 0.25 M $CaCl_2$ were added dropwise to 1 ml 2 × HEBS (50 mM Hepes, pH 7.05, 280 mM NaCl, 1.5 mM Na_2HPO_4) to form a precipitate. Cells in plates were rinsed twice with DMEM. Precipitate was added to the plate and incubated for 20 min at 37°C in 5% CO_2. DMEM containing 10% FBS and 50 mM chloroquine was added to 10 ml. After 4 h, medium was replaced with 0.5% FBS. After 32 h, PDGF-BB or PMA were added at 30 and 50 ng/ml, respectively, and further incubated for 15 h. Protein extracts were prepared and CAT enzyme activity was analyzed according to published protocols (Bignon et al., 1993; Lopata et al., 1984).

Downregulation of PKC-ζ Protein by Antisense Inhibition of Translation

Phosphorothioate DNA oligonucleotides with the sequences 5′-ATGCCCAGCAGGACC-3′ (sense 1143), 5′-GGTCCTYGCTGGGCAT-3′ (antisense 1142) and 5′-GGTCCTGCTGGGCATGCGAAAGC-3′ (antisense 1144) were synthesized by Promega (Madison, WI). Subconfluent adult human dermal fibroblasts were treated with oligonucleotides in DMEM containing 20 μg/ml DOTMA/DOPE (lipofectin, GIBCO BRL) for 6–8 h at 37°C in the presence of 5% CO_2. After this time the medium containing DOTMA was replaced by fresh medium containing appropriate oligonucleotides. After 48 h, the medium was replaced with fresh medium con-

taining the oligonucleotides with or without 30 ng/ml PDGF-BB. After incubation for 18 h, total RNA was isolated and analyzed with Northern blotting and hybridization.

Immunoprecipitation and In Vitro PKC-ζ Activity Assay

Human dermal fibroblasts treated in the presence or absence of oligonucleotides were cultured with or without PDGF-BB for 16–24 h. The in vitro PKC-ζ kinase assay was performed according to modification of a previously described procedure (Lozano et al., 1994). Briefly, cell extracts were prepared in the lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, and proteinase inhibitors) and incubated at 4°C overnight with a polyclonal anti–PKC-ζ antibody. The immune complexes were recovered by anti–rabbit IgG agarose beads (Sigma). The resulting immunoprecipitates were washed three times in cold with a buffer containing 35 mM Tris, pH 7.5, 15 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM EGTA, 25 μg/ml leupeptin, and 25 μg/ml aprotinin before mixed in a final volume of 50 μl in assay mixture (35 mM Tris, pH 7.5, 15 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$, 1 mM sodium orthovanadate, 1 mg/ml histone III-ss (Sigma), and 100 μM [γ-^{32}P]ATP with or without 280 μg/ml phosphatidylserine) and incubated at 30°C for 10 min. After reactions were stopped by the addition of 100 mM EDTA and 2 mM ATP, 1/5 vol of the supernatant was spotted onto phosphocellulose p81 papers (Whatman, Clifton, NJ) and subsequently washed with 1% phosphoric acid six times. ^{32}P incorporation was quantitated using a scintillation counter. In some experiments the remaining supernatant was analyzed by SDS-PAGE followed by autoradiography.

Immunoblotting

Human dermal fibroblasts in the presence or the absence of oligonucleotides were cultured with or without 30 ng/ml PDGF-BB for 16–24 h. Cell extracts were prepared in the lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, and proteinase inhibitors). 5 μg protein-containing cell extracts were separated on 8% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). The membranes were incubated with a blocking solution containing 1% BSA, 2% horse serum, 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h, and then incubated overnight at 4°C with polyclonal antibodies against PKC-ζ at 4 μg/ml or PKC-μ at 1 μg/ml. After incubation with horseradish peroxidase–conjugated goat anti–rabbit antibody (1:1,000 dilution; Amersham Corp., Arlington Heights, IL) in 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 (ECL; Amersham). Protein loading was controlled by blotting the same membrane with a monoclonal anti–β-tubulin at 1:1,000 dilution.

Results

PDGF-BB and PMA Induce α_2 mRNA Levels and Promoter-directed Gene Transcription

Previous studies have shown that PDGF-BB induces α_2 integrin mRNA steady-state levels in human foreskin (Ahlen and Rubins, 1994) and adult dermal (Xu and Clark, 1996) fibroblasts. The time course here showed that this induction occurs as early as 4 h after PDGF was added and peaked at 24 h (Fig. 1 *A*). PMA, a tumor promoter activating protein kinase C, also induced α_2 mRNA steady-state levels, but the steady-state levels peaked earlier than that by PDGF (Fig. 1 *B*). Maximal induction was observed at 8 h and dropped significantly by 24 h.

To determine that increased α_2 mRNA steady-state levels occurred at least in part as a consequence of transcription, transient expression assays were performed. Previously, it has been found that a region between 92 and 961 base pairs upstream of the transcription start site of human α_2 gene consists of numerous consensus enhancer elements (Zutter et al., 1994) including those PDGF- or PMA-responsive sequences, i.e., SIFE (sis-inducible factor binding element; Wagner et al., 1990), SRE (serum responsive element; Treisman, 1985; Rupprecht et al., 1993), AP-1 (Angel et al., 1987), and NF-κB–(Hansen et al., 1992) binding sites. Therefore for these assays, two α_2 promoter-CAT constructs, p$\alpha_2$92-CAT and p$\alpha_2$961-CAT, were transfected into adult human dermal fibroblasts which were then subjected to serum deprivation for 48 h followed by stimulation with PDGF and PMA for 16 h, separately or together. Cell lysates were assayed for CAT activity (Fig. 2). In multiple experiments, the p$\alpha_2$961-CAT was induced by both PDGF and PMA (Fig. 2), whereas the p$\alpha_2$92-CAT was not (data not shown). This result was consistent with transcriptional regulation directed by a PDGF- and PMA-inducible α_2 promoter.

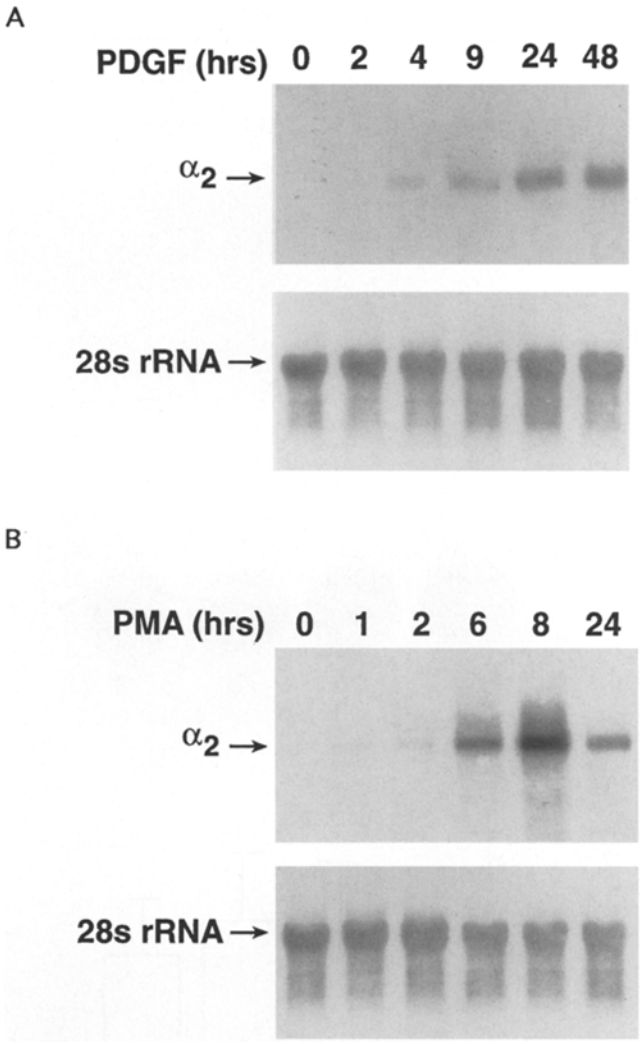

Figure 1. Time course of integrin α_2 subunit mRNA steady-state level induced by PDGF and PMA. Normal human dermal fibroblasts were cultured 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Such cultures were then stimulated by (*A*) 30 ng/ml PDGF-BB or (*B*) 50 ng/ml PMA and incubated for the time indicated. Total RNA was probed with human α_2 integrin cDNA. 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.

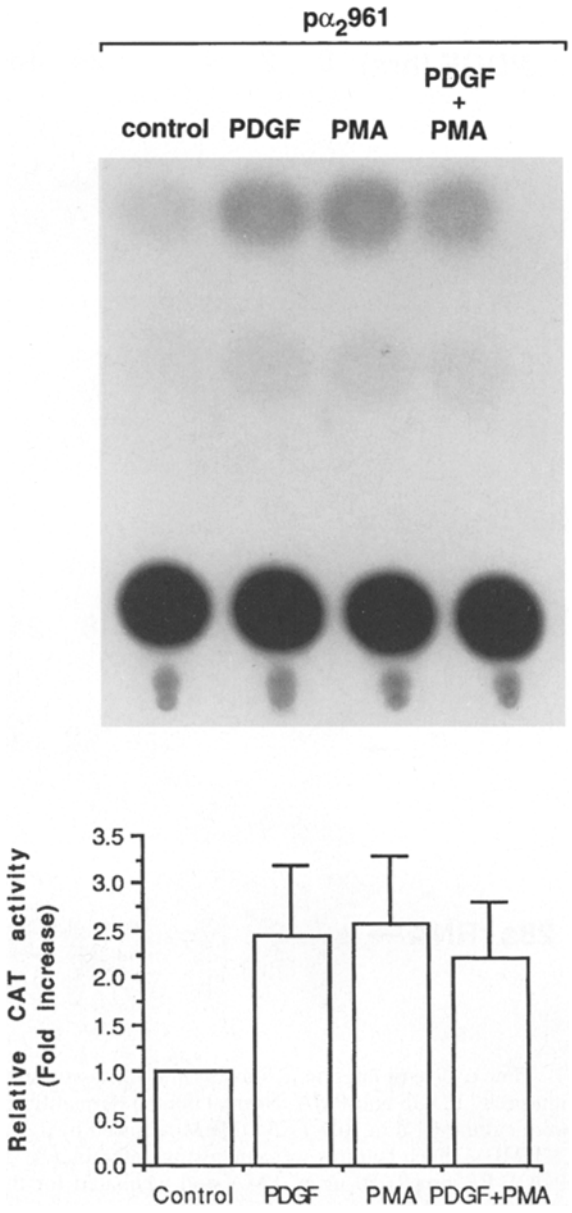

Figure 2. Induction of α_2 promoter activity by PDGF and PMA. A CAT construct, p$\alpha_2$961-CAT which contains 961 base pairs of α_2 upstream region, was transfected into normal human dermal fibroblasts. Cotransfection with RSV-β-galactosidase was used as an internal control. Transfected cells were starved for 48 h before treatment with PDGF and PMA for 16 h, separately or together, and cellular proteins were extracted. After normalization with β-galactosidase activity, CAT enzymatic activity was determined by thin layer chromatography. After autoradiography, the levels of acetylated chloramphenicol were quantified by clipping the radioactive spots from the TLC plates and by counting them in a liquid scintillation counter. The graphic results shown are the mean value ±SD of three independent experiments. Relative CAT activity is defined as the fold-increase of CAT activity generated from cells treated with various additatives compared to cells without treament.

The PDGF Induction of α_2 Integrin Expression Requires Protein Phosphorylation by Both Protein Tyrosine Kinase and Protein Kinase C

The signaling cascades from PDGF receptor stimulation to increased α_2 gene transcription have not been defined. Since the PDGF receptor is a protein tyrosine kinase, the involvement of tyrosine phosphorylation in the PDGF stimulation of α_2 mRNA was examined with a tyrosine kinase inhibitor, genistein (Akiyama and Ogawara, 1991). Fig. 3 *A* demonstrates that treatment of fibroblasts with genistein at 100 μM blocked PDGF stimulation of α_2 mRNA. PMA, a well known protein kinase C activator, was expectedly not antagonized by genistein.

The PDGF receptor, a tyrosine kinase, generates diacylglycerol upon activation which, like PMA, is theoretically a potent inducer of classic and nonclassic protein kinase C. Therefore, it was of interest to evaluate whether protein kinase C was required for PDGF induction of α_2 mRNA. Two specific protein kinase C inhibitors were used, chelerythrine (Herbert et al., 1990) and bisindolylmaleimide GF 109203X (BIM; Toullec et al., 1991). BIM inhibited both PDGF and PMA induction whereas chelerythrine had no effects on PMA induction and incompletely inhibited PDGF induction (Fig. 3 *B*). Therefore, protein kinase C is also involved in PDGF pathway for α_2 mRNA induction.

The PDGF Induction of α_2 Transcription Requires Both Protein Tyrosine Kinase and Protein Kinase C

To substantiate that protein phosphorylation is required for α_2 integrin gene expression, transient expression assay with a p$\alpha_2$961-CAT construct was performed in the presence or absence of PKC or PTK inhibitors. After transfection, human dermal fibroblasts were starved for 48 h followed by stimulation with PDGF and PMA in the presence or absence of BIM, chelerythrine, or genistein. Cell lysates were assayed for CAT activity. PDGF-induced promoter activity was completely inhibited by genistein or BIM, but only partially by chelerythrine (Fig. 4 *A*). Unlike PDGF, PMA induced the promoter activity even in the presence of genistein or chelerythrine but not BIM (Fig. 4 *B*). These results demonstrate that there is a consistency in the requirement of PKC and PTK activities between the promoter directed gene expression and the mRNA steady-state level (Fig. 3, *A* and *B*). Therefore, the phosphorylation requirement occurs at the level of promoter-directed transcription of α_2 gene.

The PDGF Induction of α_2 Expression Requires a Non–PMA-inducible Protein Kinase C

Although both PDGF and PMA use protein kinase C to induce α_2 expression, there were disparities between two activation pathways. The time course and the sensitivity to chelerythrine differed between two stimuli. In addition, PDGF induction of α_2 mRNA expression required protein synthesis while PMA stimulated expression did not (Fig. 5). Thus, we decided to examine whether the two activators actually use the same isoforms of protein kinase C. A widely used strategy is to deplete cellular PMA-inducible PKC levels by treating cell culture chronically with PMA

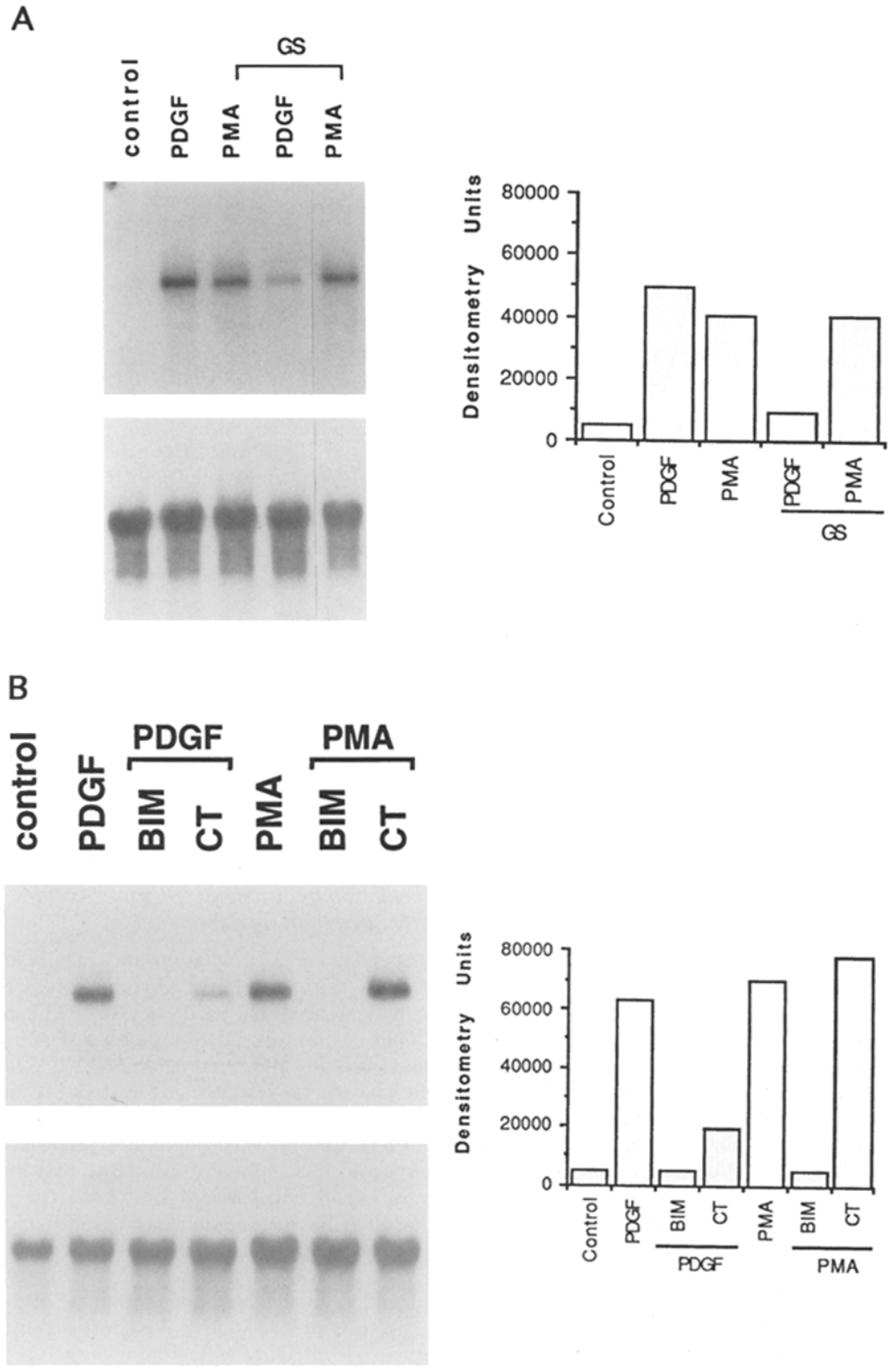

Figure 3. Inhibition of PDGF and PMA stimulation of α_2 mRNA level. Cells were treated with inhibitors for protein tyrosine kinase and protein kinase C for 2 h before addition of 30 ng/ml PDGF and 50 ng/ml PMA. Total RNA was probed with human α_2 integrin cDNA. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA. (*A*) Protein tyrosine kinase inhibitor, 100 μM genistein (GS) and (*B*) protein kinase C inhibitors, 5 μM bisindolylmaleimide GF 109203X (BIM) and 1 μM chelerythrine (CT).

(Larrodera et al., 1990). This approach depletes PKC isoforms sensitive to DAG/PMA activation. Thus, we exposed quiescent human fibroblast cultures to PMA (300 ng/ml) for 48 h. α_2 mRNA steady-state levels were then determined following the addition of either PDGF or PMA. Although PMA was unable to induce α_2 mRNA in cells after PMA-inducible PKC depletion, PDGF promoted a potent response which was even higher than in cells not pretreated with PMA (Fig. 6). These results strongly suggest that, although PMA is capable of activating α_2 mRNA, PDGF uses an atypical PKC isoform not activated by PMA.

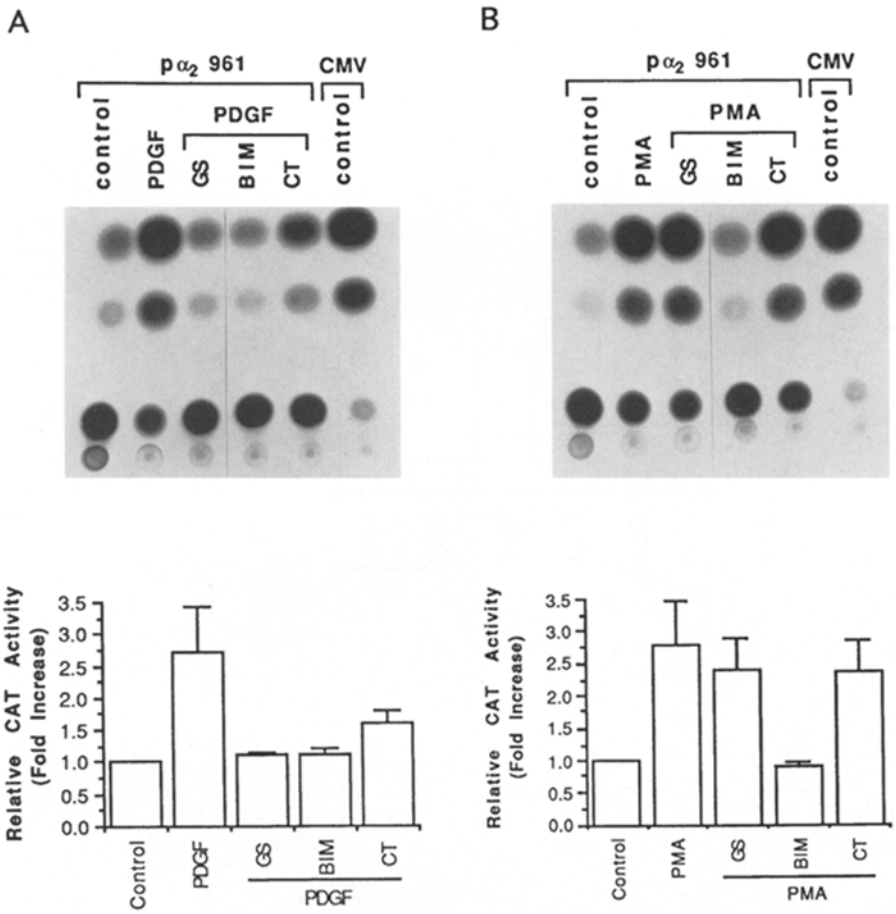

Figure 4. Inhibition of PDGF stimulation of α_2 promoter activity. Fibroblasts were cotransfected with p$\alpha_2$961-CAT and RSV-β-galactosidase and 46 h later treated with PTK and PKC inhibitors for 2 h before stimulation with (*A*) 30 ng/ml PDGF and (*B*) 50 ng/ml PMA for 16 h. CAT activity was determined after normalization with β-galactosidase activity. The transfection with cytomegalovirus-CAT (CMV) which contains strong viral promoter was performed in parallel. The assay shown is representative of three similar experiments. The graphic quantification is the mean value of three independent assays ±SD. *GS*, 100 μM genistein; *BIM*, 5 μM bisindolylmaleimide GF 109203X; *CT*, 1 μM chelerythrine.

PDGF Induces Activation of PKC-ζ

One of the atypical PKC isoforms, ζ, has been proposed in PDGF signal transduction by both direct and indirect evidence. Dominant kinase-defective mutants of PKC-ζ impair PDGF activation of stromelysin gene promoter directed transcription in NIH-3T3 fibroblasts (Sanz et al., 1994). Products of several PDGF-inducible enzymes are PKC-ζ activators, such as phosphatidylinositol 3,4,5-triphosphate (PIP_3), phosphotidic acid, and phosphatidylcholine hydrolysis products (Nakanishi et al., 1993; Dominguez et al., 1992). The involvement of PKC-ζ in PDGF-induced α_2 integrin expression was therefore considered as a possibility. To assess direct correlation between PDGF and PKC-ζ activation, the PDGF-stimulated PKC-ζ kinase activity was assayed in vitro with fibroblast extracts. Human dermal fibroblasts were treated with PDGF in the presence or absence of BIM. PKC-ζ present in the cell extracts was immunoprecipitated with a polyclonal antibody. Hinstone III-ss was phosphorylated by kinase activities associated with the immunoprecipitates. PDGF increased PKC-ζ kinase activity (Fig. 7 and Table I). The presence of BIM, a specific PKC inhibitor, reduced the PDGF-induced PKC-ζ activity to almost basal levels. Phosphatidylserine, a PKC activator, induced the kinase activity similar to PDGF. Therefore, PKC-ζ appears to become activated, as determined by kinase activity associated with PKC-ζ immunoprecipitates, in response to stimulation with PDGF in human fibroblasts.

PKC-ζ Downregulation by Antisense Oligonucleotides Inhibits PDGF Induction of α_2 Expression

The correlation between PKC activation and PDGF induction of α_2 mRNA as revealed by PKC inhibitors presented convincing circumstantial evidence for PKC involvement in PDGF signal transduction pathway leading to α_2 expression. Given that PMA-inducible PKCs had no effect on the pathway, it suggests the involvement of an atypical isoform. To test this possibility, a more specific inhibitor of these PKC isoforms was sought. One approach is to deplete a specific PKC subtype through translation inhibition by antisense oligonucleotides.

Antisense phosphorothioate oligonucleotides, complementary to the 5′-end of the PKC-ζ messenger starting at the translation initiation codon (Barbee et al., 1993), were synthesized and added to the medium of human fibroblasts to a concentration of 2.5–4.0 μM. We chose an antisense target sequence located at the beginning of the open reading frame of PKC-ζ cDNA because this GC-rich site is in a nonconserved variable region of PKC family (V1 region; Nishizuka, 1992) which allows the design of a sequence effectively unique to PKC-ζ when compared with cDNA sequences of other PKCs. Furthermore, this RNA site has been effectively targeted by antisense oligonucleotides in murine T lymphocytes (Gomez et al., 1995). "Sense" oligonucleotides were used as control. The depletion of PKC-ζ protein levels by antisense inhibition of translation was monitored by kinase activity of PKC-ζ im-

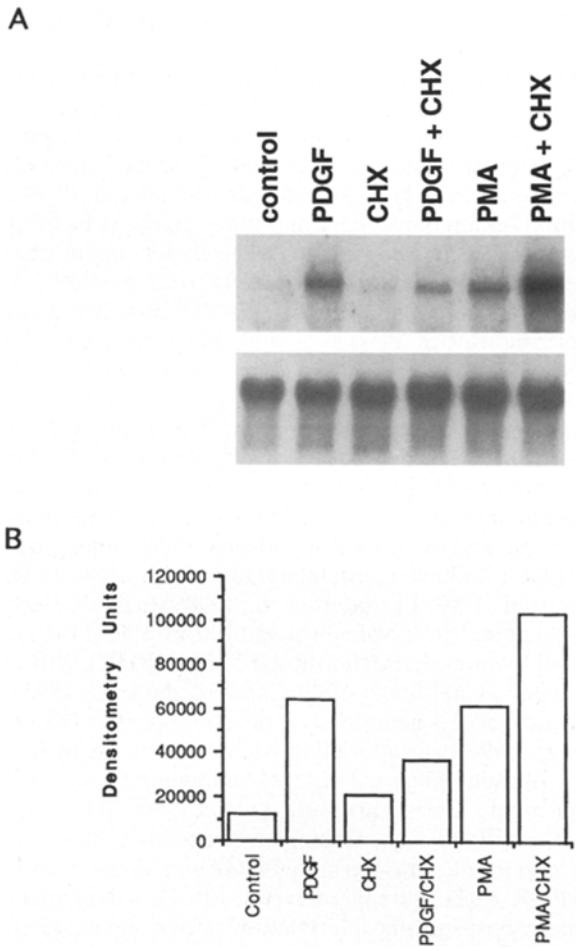

Figure 5. Protein synthesis requirement of PDGF and PMA stimulation of α_2 mRNA level. Cells were treated with protein synthesis inhibitor cycloheximide (CHX) at 7.5 μg/ml for 15 min before addition of 30 ng/ml PDGF and 50 ng/ml PMA. Total RNA was probed with human α_2 integrin cDNA. Equal loading was 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.

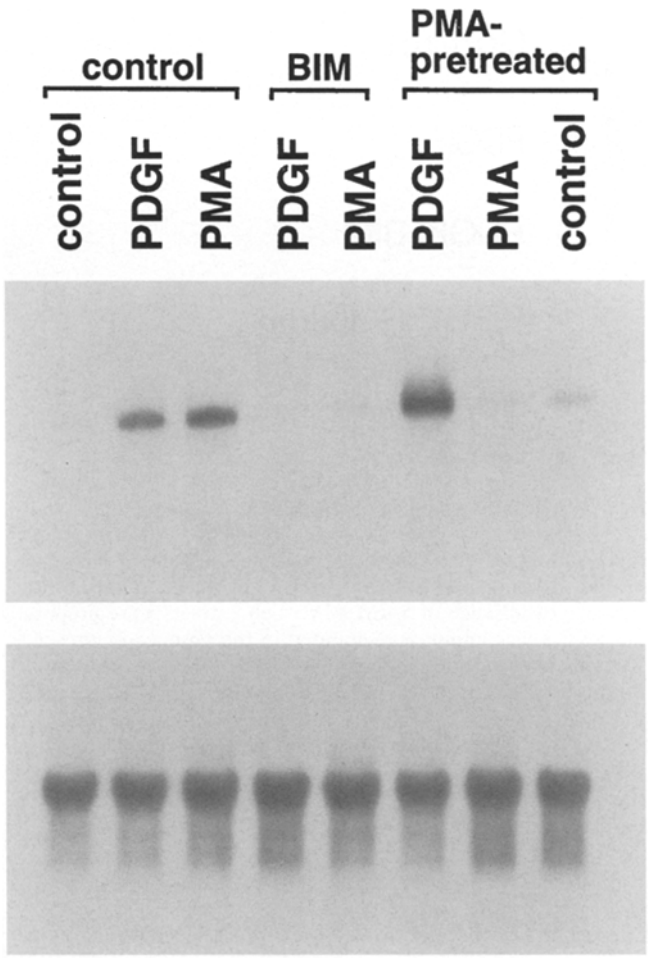

Figure 6. Stimulation of α_2 mRNA levels by PDGF and PMA after chronic treatment of fibroblasts with PMA. Quiescent human dermal fibroblasts either untreated, or treated with BIM for 2 h, or chronically incubated (48 h with PMA [300 ng/ml]) were stimulated with PDGF (30 ng/ml) or PMA (50 ng/ml) for 16 h. Total RNA was probed with human α_2 integrin cDNA. 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.

munoprecipitates (Fig. 8 *A*) and by Western blotting (Fig. 8 *B*) with antibody specific for human PKC-ζ. Incubation of subconfluent human dermal fibroblasts for 48 h in medium containing antisense oligonucleotides, but not sense oligonucleotides, significantly downregulated PKC-ζ associated kinase activity and protein level in a dose-dependent manner (Fig. 8, *A* and *B*). The possibility that such antisense inhibition may be random downregulation of cellular proteins was examined by reprobing the membrane with another PKC isotype, μ (Fig. 8 *B*). The unchanged amount of cellular PKC-μ even at the maximal dose of antisense oligonucleotides used (4 μM) confirmed the specificity of the antisense inhibition of PKC-ζ. In addition, total PKC enzymatic activity was not substantially altered by antisense oligonucleotides to PKC-ζ (data not shown). Such cells were treated with PDGF for 16–24 h and harvested for analysis of α2 mRNA expression. Antisense treatment inhibited PDGF induction of α_2 expression up to 70% (Fig. 9, *A* and *B*), consistent with the results obtained when the two PKC inhibitors, chelerythrine and BIM, were used (Fig. 3 *B*). Therefore, PKC-ζ is involved in PDGF induction of the α_2 integrin subunit. Previously, we have found that PDGF increased α_5 and α_3 integrin mRNA levels (Xu and Clark, 1996). To examine whether PKC-ζ is required for α_2 specifically or for integrin mRNAs in general, the same RNA blot was reprobed for α_5 and α_3. Interestingly, the antisense treatment also downregulated another PDGF-induced integrin messenger RNA, α_5, but not α_3, indicating that PKC-ζ activity is required for PDGF-induced mRNA levels of selective integrins.

Discussion

We report here that PDGF and PMA induced α_2 integrin mRNA level through promoter-directed transcription. By using inhibitors for PTK and PKC, we further show that this PDGF activation requires both kinase activities.

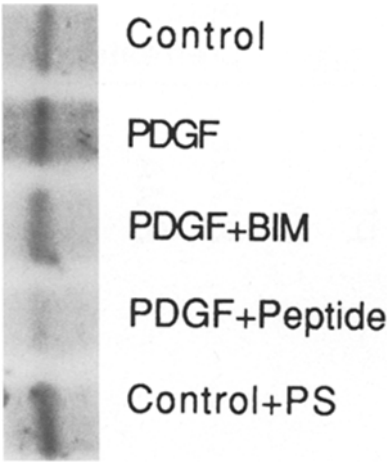

Figure 7. Increased phosphorylating activity in immunoprecipitates of PKC-ζ from PDGF-stimulated human dermal fibroblasts. Subconfluent normal human dermal fibroblasts were either untreated or stimulated for 18 h with 30 ng/ml PDGF-BB in the presence or absence of 5 μM BIM. Cell extracts were prepared and PKC-ζ was immunoprecipitated in the absence or presence of competing peptide. Kinase reactions were performed for 10 min at 30°C either in the absence or presence of 280 μg/ml of phosphatidylserine (PS) as described in Materials and Methods. Histon III-ss (Sigma) phosphorylation was determined by autoradiography following separation of proteins by 10% SDS-PAGE. The film was exposed for 1.5 h. Quantitation was determined by liquid scintillation counting of each aliquot as shown in Table I.

Furthermore, specific depletion of PKC-ζ proteins by antisense oligonucleotide-mediated translational inhibition blocked PDGF induction of the same gene, as did the chemical inhibitors of PKC.

Many investigators have shown that PDGF signal transduction pathways use protein tyrosine phosphorylation as their first step of activation with two exceptions, the immediate early genes, *c-fos* and *egr-1* (Lee and Donoghue, 1991; Mundschau and Faller, 1995; Mundschau et al., 1994). PKC and mitogen-activated protein (MAP) kinase are often effectors of PDGF pathways that have direct impact on gene transcription. PKC is involved in PDGF induced expression of the slow immediate genes JE/MCP-1 and vascular endothelial growth factor (VEGF) (Finkenzeller et al., 1992), and stromelysin, a late responding gene (Sanz et al., 1994).

Table I. PDGF-induced Increase of PKC-ζ Activity

Conditions	Counts per minute
Control	1,568 ± 188
PDGF	6,490 ± 653
PDGF + BIM	1,850 ± 347
PDGF + peptide	665 ± 102
Control + PS	4,821 ± 1,301

Cell lysates from fibroblasts untreated or treated with PDGF for 18 h were immunoprecipitated with polyclonal antibody against PKC-ζ in the presence or absence of a synthetic peptide the antibody was raised against. The immunoprecipitates were incubated with histone III-ss for 10 min at 30°C in kinase assay buffer. Unless specified, the reactions were carried out in the absence of phosphatidylserine (PS). The kinase activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone III-ss as described in Materials and Methods. The results represent the mean ±SD of three independent experiments. One of the experiments was shown as Fig. 7.

In the case of α_2 gene activation, the fact that PKC-ζ, instead of PMA-sensitive PKC isoforms, is required for PDGF gene induction in human skin fibroblasts is preceded by similar observations in PDGF-induced stromelysin expression in NIH 3T3 fibroblasts (Sanz et al., 1994). In addition, sphingomyelinase and tumor necrosis factor α (TNFα)–induced nuclear factor (NF)-κB–dependent promoter activation (Lozano et al., 1994), IL-2–mediated T cell proliferation (Gomez et al., 1995), PDGF-induced mitogenic responses in swiss 3T3 cells and insulin-induced maturation of Xenopus oocytes (Berra et al., 1993) are all PKC-ζ–dependent processes.

Although it is not well understood, PKC-ζ, an atypical PKC isoform that does not have diacylglycerol (DAG) and phorbol ester binding domain, is reportedly activated by phosphatidylinositol 3,4,5-triphosphate (PIP_3), phosphotidic acid, and hydrolysis of phosphatidylcholine, products of PDGF-inducible enzymes (Dominguez et al., 1992; Homma et al., 1993; Larrodera et al., 1990; Ma et al., 1994; Nakanishi et al., 1993; Nakanishi and Exton, 1992). PIP_3 is generated by phosphatidylinositol 3-kinase (PI3K) activation (Auger et al., 1989; Valius and Kazlauskas, 1993); phosphatidic acid is generated by phospholipase D (PLD) (Lee et al., 1994); and phosphatidylcholine (PC) is hydrolyzed by phospholipase C (PLC) (Dominguez et al., 1992, Homma et al., 1993; Larrodera et al., 1990; Ma et al., 1994). Thus, there is increasing evidence that PKC-ζ is part of important pathway(s) in PDGF signaling. Activation of PI3K, PLD, and several types of PLCs is dependent on protein tyrosine phosphorylation (Chen et al., 1994; Homma et al., 1993; Lee et al., 1994; Rodriguez-Viciana et al., 1994). The data presented here that inhibitors for PKC, as well as PTK, interfered PDGF signaling pathway toward α_2 expression is consistent with these observations.

Transfection of human fibroblasts with a p$\alpha_2$961-CAT construct demonstrated that this PDGF-induced second messenger pathway has a direct link to transcriptional activation of the α_2 gene (Fig. 4). How might the activation of PKC-ζ lead to α_2 gene transcription? Although downstream targets of PKC-ζ are yet to be completely elucidated, possibilities include direct or indirect control of transcription factors. PKC-ζ has been shown to regulate NF-κB activity through its ability to phosphorylate the NF-κB inhibitor protein, IκB (Diaz-Meco et al., 1994*b*). Therefore activation of PKC-ζ can lead to NF-κB released from its inhibitor protein and subsequently translocated into nucleus where it would activate a group of genes that have appropriate responsive elements. Another observation that is of interest is the nuclear presence of PKC-ζ in rat brain (Hagiwara et al., 1990) and human dermal fibroblasts (data not shown). Recently, two other PKC isoforms, δ and ε, were also found present in nuclei isolated from rat cardiac myocytes (Ventura et al., 1995). Although its biochemical significance has yet to be determined, the nuclear location of PKC-ζ suggests it may directly phosphorylate transcription factors in nuclei. Alternatively, PKC-ζ may indirectly regulate transcription factors. Phorbol ester–activated PKC phosphorylates and activates Raf-1, which in turn activates MAPK through a protein kinase cascade (Kolch et al., 1993; Marquardt et al., 1994;

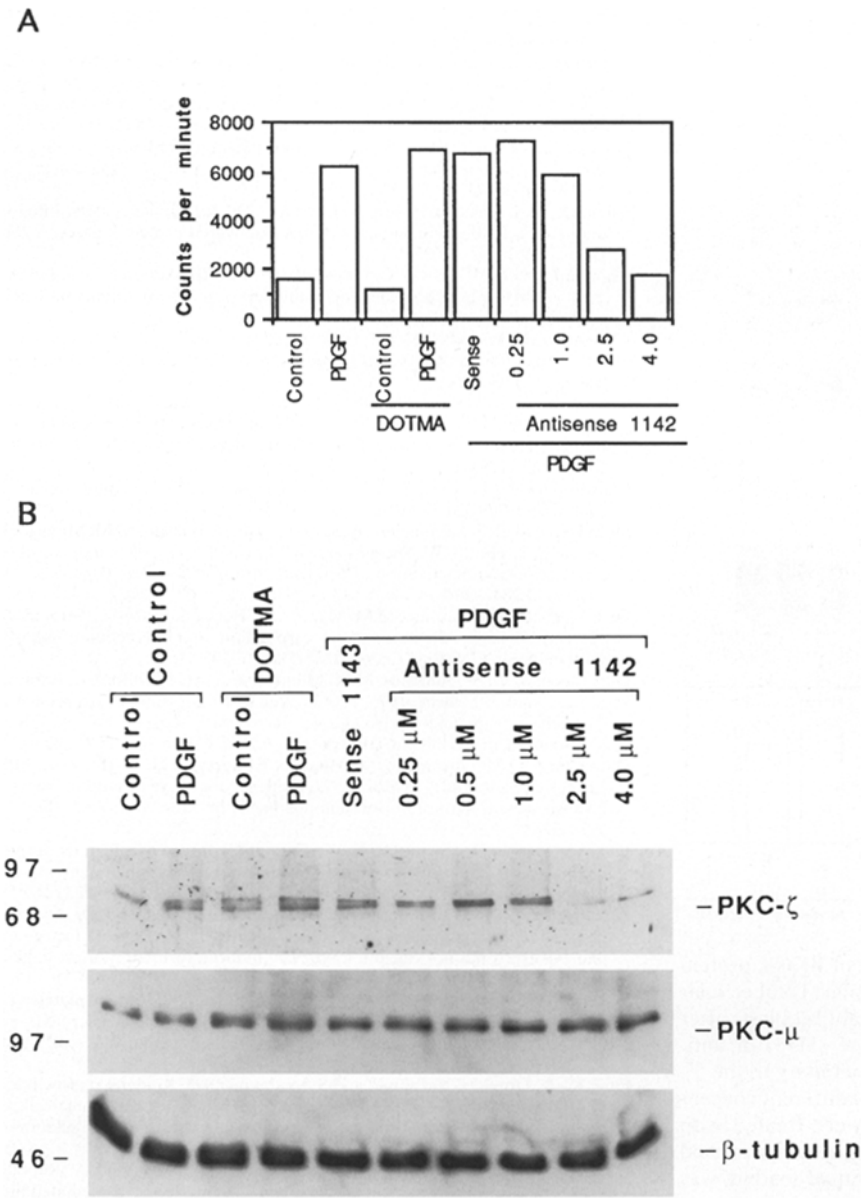

Figure 8. Specific downregulation of PKC-ζ activity and protein levels by antisense inhibition of translation. Subconfluent normal dermal human fibroblasts were either untreated or treated with sense (1143) or antisense (1142) oligonucleotides at different concentration in DMEM containing 20 μg/ml DOTMA for 6–8 h. After this time the medium containing DOTMA was replaced by fresh medium containing appropriate oligonucleotides which were replaced 48 h later by fresh oligonucleotides before stimulation with 30 ng/ml PDGF 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*) In vitro kinase activity in immunoprecipitates of PKC-ζ. The kinase activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone III-ss as described in Materials and Methods. (*B*) Western blot detection of PKC-ζ and PKC-μ in total cell extracts. Detection with a monoclonal antibody against β-tubulin was used as a control for protein loading.

Sozeri et al., 1992). Substrates of mitogenic-activated protein kinase (MAPK) include several transcription factors such as Jun and Fos. PKC-ζ also can activate MAPK through a different mechanism. It is associated with and activates MAPK kinase (MEK) and MAPK complex in vitro (Diaz-Meco et al., 1994*a*). Raf protein, however, is not associated with or phosphorylated by PKC-ζ (Diaz-Meco et al., 1994*b*).

The first 961 base pairs (bp) of the α_2 promoter region consist of many regulatory elements including NF-κB and AP-1–binding sequences (Zutter et al., 1994). This is consistent with PKC-ζ's ability to generate NF-κB and AP-1–binding activity. In addition, there have been reports that new PDGF responsive elements are located in both the slow immediate gene JE/MCP-1 and the late gene stromelysin (Freter et al., 1995; Sanz et al., 1994). The nuclear factor binding to the PDGF responsive element of JE/MCP-1 gene is a serine/threonine phosphoprotein and nuclear extracts from cells overexpressing PKC-ζ give DNA binding to the PDGF responsive element of stromelysin gene comparative to that induced by PDGF. It is thus possible that undefined DNA regulatory elements might be responsible for the phosphorylation-regulated α_2 gene expression. Detailed mapping of α_2 promoter regulatory sequences in response to PDGF is currently under way.

Dermal fibroblasts appear to use this PDGF second messenger pathway to induce a gene set that includes α_2 integrin subunit, stromelysin-1 (Sanz et al., 1994) and metalloproteinase-1 (MMP-1; data not shown). The proteins translated from these genes are essential for fibroblast interaction with interstitial collagen matrices.

We thank Dr. Marcia Simon, Department of Dermatology (SUNY at Stony Brook) for human adult dermal fibroblasts; Dr. Lorne Taichman, Department of Oral Biology and Pathology (SUNY at Stony Brook) for the RSV-β-gal plasmid; Dr. Charles Hart, ZymoGenetics (Seattle, WA) for PDGF-BB, and Dr. Yoshikazu Takada of The Scripps Institute (San Diego, CA) for human α_2 and α_3 cDNA probes.

Funding for this work was provided by National Institutes of Health grant AG10114309 to R.A.F. Clark. J. Xu is a postdoctoral fellow sup-

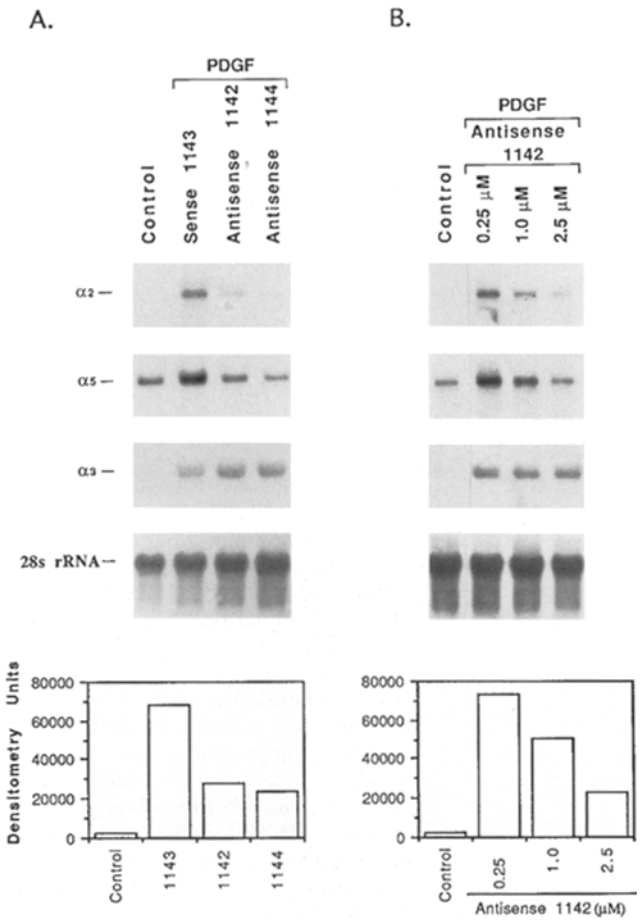

Figure 9. Antisense-mediated downregulation of PKC-ζ protein inhibits PDGF induction of integrin α_2 expression. Total cellular RNA was extracted from normal human dermal fibroblasts after incubation in the presence or absence of sense (1143) or antisense (1142 and 1144) oligonucleotides complementary to the 5′-end of the PKC-ζ transcript at 2.5 μM (*A*) or at different concentrations (*B*). Before RNA harvest, cultures were treated with PDGF (30 ng/ml) for 16 h. Total RNA was sequentially probed with human α_2, α_5, and α_3 integrin 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.

ported by funding from the Dermatology Foundation and the School of Medicine (SUNY at Stony Brook).

Received for publication 25 March 1996 and in revised form 14 June 1996.

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