PDGF Induction of α₂ 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 α₂β₁ 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 α₂ 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 α₂ 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 α₂ mRNA levels up to 70%, indicating PKC-ζ, a non-PMA-sensitive PKC isoform, is a component of the PDGF stimulatory pathway for α₂ mRNA synthesis. A 961-base pair (bp) upstream region of α₂ 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 α₂ promoter fragment/CAT construct, indicating that the phosphorylation requirement occurred at α₂ promoter-directed transcription level. Therefore, we propose that PDGF-stimulatory pathway of α₂ 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 α₁–α₁0 and α₅ (Haas and Plow, 1994; Hynes, 1992). Among them, α₂β₁ 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 α₂β₁ 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 α₂ integrin subunit expression include platelet-derived growth factor (PDGF) (Ahlen and Rubins, 1994; Xu and Clark, 1996), transforming growth factor-β (Riikonen et al., 1995), epidermal growth factor (Fuji 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-

1. Abbreviations used in this paper: DAG, diacylglycerol; MAPK, mitogen-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 *egf-I* (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* (*JEMCP-1*) (Freter et al., 1995), and late genes such as stromelysin (Diaz-Meco et al., 1991) and interstitial collagenase (Circello et al., 1991). However, the events linking early second messenger activation to 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, β, βII, and γ depends on Ca²⁺ and phorbol ester/diacylglycerol (DAG) for activity. The nonclassic group containing isoforms δ, ε, θ, Ψ, and χ is phorbol ester/DAG-dependent but does not require Ca²⁺. 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 α₂ 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 α₂ integrin gene expression. Furthermore, human dermal fibroblasts were transfected with α₂ promoter region/CAT constructs to establish the link between PDGF-induced kinase activity and α₂ 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 α₂ 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, Depart-
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 immunocomplexes 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 MgCl₂, 1 mM MnCl₂, 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 MgCl₂, 1 mM MnCl₂, 0.5 mM EGTA, 0.1 mM CaCl₂, 1 mM sodium orthovanadate, 1 mg/ml histone III-s (Sigma), and 100 μM [γ-32P]ATP with or without 280 μg/ml phosphatidylinerine) 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 papers (Whatman, Clifton, NJ) and subsequently washed with 1% phosphoric acid six times. 32P 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 α₂ mRNA Levels and Promoter-directed Gene Transcription**

Previous studies have shown that PDGF-BB induces α₂ 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 α₂ 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 α₂ 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 α₂ 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 α₂-promoter-CAT constructs, pα₂-92-CAT and pα₂-961-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 pα₂-961-CAT was induced by both PDGF and PMA (Fig. 2), whereas the pα₂-92-CAT was not (data not shown). This result was consistent with transcriptional regulation directed by a PDGF- and PMA-inducible α₂ promoter.
The PDGF Induction of $\alpha_2$ Integrin Expression Requires Protein Phosphorylation by Both Protein Tyrosine Kinase and Protein Kinase C

The signaling cascades from PDGF receptor stimulation to increased $\alpha_2$ 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 $\alpha_2$ 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 mM blocked PDGF stimulation of $\alpha_2$ 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 $\alpha_2$ mRNA. Two specific protein kinase C inhibitors were used, chelerythrine (Herbert et al., 1990) and bisindoylmaleimide 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 $\alpha_2$ mRNA induction.

The PDGF Induction of $\alpha_2$ Transcription Requires Both Protein Tyrosine Kinase and Protein Kinase C

To substantiate that protein phosphorylation is required for $\alpha_2$ integrin gene expression, transient expression assay with a $p\alpha_{2961}$-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 $\alpha_2$ gene.

The PDGF Induction of $\alpha_2$ Expression Requires a Non–PMA-inducible Protein Kinase C

Although both PDGF and PMA use protein kinase C to induce $\alpha_2$ 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 $\alpha_2$ 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 \( \alpha_2 \) 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 \( \alpha_2 \) 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 \( \mu \)M genistein (GS) and (B) protein kinase C inhibitors, 5 \( \mu \)M bisindolylmaleimide GF 109203X (BIM) and 1 \( \mu \)M 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. \( \alpha_2 \) mRNA steady-state levels were then determined following the addition of either PDGF or PMA. Although PMA was unable to induce \( \alpha_2 \) 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 \( \alpha_2 \) mRNA, PDGF uses an atypical PKC isoform not activated by PMA.
**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 (PIP3), phosphotidic acid, and phosphatidylinosine hydrolysis products (Nakanishi et al., 1993; Dominguez et al., 1992). The involvement of PKC-ζ in PDGF-induced α2 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. Histone III-s 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 α2 Expression**

The correlation between PKC activation and PDGF induction of α2 mRNA as revealed by PKC inhibitors presented convincing circumstantial evidence for PKC involvement in PDGF signal transduction pathway leading to α2 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 PKCs 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 (Gómez 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-
Protein synthesis requirement of PDGF and PMA stimulation of α2 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 α2 integrin cDNA. 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.

Stimulation of α2 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 α2 integrin cDNA. 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.

Figure 5. Protein synthesis requirement of PDGF and PMA stimulation of α2 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 α2 integrin cDNA. 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.

Figure 6. Stimulation of α2 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 α2 integrin cDNA. 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.

Discussion

We report here that PDGF and PMA induced α2 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.
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) (Finkenfelder et al., 1992), and stromelysin, a late responding gene (Sanz et al., 1994).

In the case of α2 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 (PIP3), phosphotidic acid, and hydrolysis of phosphatidylycholine, products of PDGF-inducible enzymes (Dominguez et al., 1992; Homma et al., 1993; Larrodera et al., 1990; Ma et al., 1994; Nakashishi et al., 1993; Nakashishi and Exton, 1992). PIP3 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 phosphatidylycholine (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 α2 expression is consistent with these observations.

Transfection of human fibroblasts with a pα2961-CAT construct demonstrated that this PDGF-induced second messenger pathway has a direct link to transcriptional activation of the α2 gene (Fig. 4). How might the activation of PKC-ζ lead to α2 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., 1994b). 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; 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-s for 10 min at 30°C in kinase assay buffer. Unless specified, the reactions were carried out in the absence of phosphatidyserine (PS). The kinase activity was determined by measuring the incorporation of 32P from [γ-32P]ATP into histone III-s 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.

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 phosphatidyserine (PS) as described in Materials and Methods. Histone III-s (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.
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., 1994a). Raf protein, however, is not associated with or phosphorylated by PKC-ζ (Diaz-Meco et al., 1994b).

The first 961 base pairs (bp) of the α2 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 α2 gene expression. Detailed mapping of α2 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 α2 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.

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with human c~2, tx 5, and c~3 integrin cDNAs. Equal loading was
confirmed by hybridization of the same blot with 32p-la-
trations (B). Before RNA harvest, cultures were treated with
end of the PKC-c transcript at 2.5 ~M (A) or at different concen-
tations (1142 and 1144) oligonucleotides complementary to the 5'-
RNA was extracted from normal human dermal fibroblasts after
incubation in the presence or absence of sense (1143) or anti-
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Figure 9. Antisense-mediated downregulation of PKC-c protein
inhibits PDGF induction of integrin a2 expression. Total cellular
RNA was extracted from normal human dermal fibroblasts after
incubation in the presence or absence of sense (1143) or anti-
sense (1142 and 1144) oligonucleotides complementary to the 5’-
end of the PKC-c transcript at 2.5 ~M (A) or at different concentra-
tions (B). Before RNA harvest, cultures were treated with
PDGF (30 ng/ml) for 16 h. Total RNA was sequentially probed
with human a2, a5, and a3 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-la-
belled probe for 28S ribosomal RNA.

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