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α2(I) collagen gene regulation by protein kinase C signaling in human dermal fibroblasts

Masatoshi Jinnin, Hironobu Ihn*, Kenichi Yamane, Yoshihiro Mimura, Yoshihide Asano and Kunihiko Tamaki

Department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

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ABSTRACT

We investigated the mechanisms by which protein kinase C (PKC) regulates the expression of the α2(I) collagen gene in normal dermal fibroblasts. Reduction of PKC-α activity by treatment with Gö697-6 or by overexpression of a dominant negative (DN) mutant form decreased α2(I) collagen gene expression. This decrease required a sequence element in the collagen promoter that contains Sp1/Sp3 binding sites. Reduction of PKC-δ activity by rottlerin or overexpression of DN PKC-δ also decreased α2(I) collagen gene expression. This effect required a separate sequence element containing Sp1/Sp3-binding sites and an Ets-binding site. In both cases, point mutations within the response elements abrogated the response to PKC inhibition. Forced overexpression of Sp1 rescued the PKC inhibitor-mediated reduction in collagen protein expression. A DNA affinity precipitation assay revealed that inhibition of PKC-δ by rottlerin increased the binding activity of endogenous Fli1 and decreased that of Ets1. On the other hand, TGF-β1, which increased the expression of PKC-δ, had the opposite effect, increasing the binding activity of Ets1 and decreasing that of Fli1. Our results suggest that PKC-δ is involved in the regulation of the α2(I) collagen gene in the presence or absence of TGF-β. Alteration of the balance of Ets1 and Fli1 may be a novel mechanism regulating α2(I) collagen expression.

INTRODUCTION

Systemic sclerosis or scleroderma is an acquired disorder which typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack and vascular damage, leading to the activation of fibroblasts and disturbed interactions with different components of the extracellular matrix (ECM) (1,2). Thus, abnormal scleroderma fibroblasts which are responsible for fibrosis may develop from a subset of cells that have escaped from normal control mechanisms (3,4). However, despite recent advances in understanding the regulation of collagen gene expression, the mechanisms responsible for the pathologic increase in the expression of collagen genes in scleroderma have not been elucidated.

Fibroblasts from affected scleroderma skin cultured *in vitro* produce excessive amounts of various collagens, mainly type I and type III collagens (5,6), and display increased transcription of the corresponding genes (7,8). Many of the characteristics of scleroderma fibroblasts resemble those of normal fibroblasts stimulated by transforming growth factor (TGF)-β1 (9,10), suggesting that the activation of dermal fibroblasts in scleroderma may be a result of stimulation by TGF-β signaling (11,12). Thus, the inhibition of TGF-β signaling is thought to be one of the most reliable approaches to the treatment of scleroderma, and there have been several reports that such an inhibition can decrease collagen expression *in vivo* or *in vitro* (13,14).

Jimenez *et al*. recently demonstrated that the inhibition of protein kinase C (PKC)-δ by a specific inhibitor of PKC-δ rottlerin or by the overexpression of the dominant negative mutant form (DN) of PKC-δ suppressed the expression of the αI(I) and αI(III) collagen genes in both normal and scleroderma dermal fibroblasts *in vitro*, without affecting the expression of other genes examined (15). Furthermore, they showed that scleroderma cells display higher levels of immunoreactive PKC-δ protein using immunofluorescence microscopy and immunoblotting. Their results indicate that PKC-δ may be involved in the regulation of collagen genes in normal and scleroderma fibroblasts, and suggest that the inhibition of this signaling pathway may be another therapeutic approach for

*To whom correspondence should be addressed. Tel: +81 3 3815 5411; Fax: +81 3 3814 1503; Email: IN-DER@h.u-tokyo.ac.jp

this disease. However, the mechanisms by which collagen gene expression is regulated by PKCs in human dermal fibroblasts remain unclear.

In this study, we investigated the molecular mechanisms regulating the expression of the α2(I) collagen gene by PKCs in normal dermal fibroblasts *in vitro*, and analyzed the association between PKCs and stimulation with TGF-β.

MATERIALS AND METHODS

Reagents

Calphostin C, rottlerin and Gö6976 were purchased from Calbiochem (La Jolla, CA). Anti-type I collagen-UNLB was obtained from SouthernBiotech (Birmingham, AL) (16–18). Actinomycin D and the antibody for β-actin or HA were from Sigma (St Louis, MO). Anti-PKC-α, PKC-δ, Sp1, Sp3, polyclonal Ets1 and polyclonal Fli1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKC-δantibody was from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-phosphoserine specific antibody was from Zymed Laboratories (San Francisco, CA). Mouse anti-phosphothreonine and anti-phosphoserine antibodies were from Biomedia (Foster City, CA). Mouse monoclonal antibodies for Ets1 and Fli1 were from US Biological (Swampscott, MA) and BD Pharmingen (San Diego, CA), respectively. Recombinant human TGF-β1 was from R & D systems (Minneapolis, MN). FuGENE 6 was obtained from Roche Diagnostics (Indianapolis, IN).

Cell cultures

Fibroblasts were obtained by skin biopsy from healthy donors. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 75 cm^2 culture flasks in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 50 μg/ml gentamycin, as described previously (19,20). Monolayer cultures were maintained at 37°C in 5% CO_2 in air. Fibroblasts between the third and sixth subpassages were used for experiments.

Cell lysis and immunoblotting

Human dermal fibroblasts were cultured until they were confluent, then the medium was collected. Remaining cells were washed with cold phosphate-buffered saline (PBS) twice and lysed in lysis buffer [5 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide (NaN_3), 0.1% sodium dodecyl sulfate, 1 μg/ml aprotinin, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. Aliquots of the conditioned medium (normalized for cell numbers) or cell lysate (normalized for protein concentrations as measured by the Bio-Rad reagent) were separated using SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h and incubated overnight at 4°C with antibody for type I collagen, HA or β-actin. The membranes were washed in Tris-buffered saline (TBS) and 0.1% Tween-20, incubated with secondary antibody, and washed again. The detection was performed using the Enhanced Chemiluminescence Detection system (Amersham, Arlington Heights, IL).

To examine the amounts of immunoreactive PKC isozyme α and δ in the cell lysates, confluent cells were washed with PBS and lysed using lysis buffer (15). The Triton X-100-supernatants were obtained by centrifuge. Aliquots containing 20 μg of lysate were electrophoresed on polyacrylamide gels. Anti-PKC-α or anti-PKC-δ antibody (Upstate Biotechnology) was used as the primary antibody.

To examine the amounts of immunoreactive Sp1, Ets1 or Fli1 in the cell lysates, confluent cells were washed with PBS and lysed using lysis buffer containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM sodium fluoride, 1 mM PMSF, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin. Polyclonal IgG anti-Sp1, Ets1 or Fli1 antibody was used.

RNA preparation and northern blot analysis

Total RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform method and analyzed by northern blotting, as described previously (19,21). RNA was subjected to electrophoresis on 1% agarose/formaldehyde gels and blotted onto nylon filters (Roche Diagnostics, Indianapolis, IN). The filters were UV cross-linked, prehybridized, and sequentially hybridized with cDNA probes. The following cDNA probes were used: human α2(I) collagen Pst I-Hind III fragments and GAPDH Hind III-Not I fragments. The membranes were then washed and exposed to X-ray film.

Immunoprecipitation

Levels of Phosphorylated Sp1 were examined by immunoprecipitation using anti-Sp1 or rabbit anti-phosphoserine specific antibodies as described previously (22). The same membrane was then stripped and reprobed with anti-Sp1 antibody to show the total amount of Sp1.

To examine the levels of phosphorylated Ets1 or Fli1, anti-polyclonal Ets1 or Fli1 antibody was used as described previously (23). The immunocomplexes were resolved by PAGE, transferred onto a membrane, and incubated with mouse anti-phosphothreonine or anti-phosphoserine antibody. As a loading control, immunoblotting was also performed using mouse anti-monoclonal Ets1 or Fli1, respectively.

Immunofluorescence

Fibroblasts were grown in 4-well LAK TEK chambers (Nunc, Naperville, IL) to subconfluence as described above. After 24 h of serum starvation, the cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in PBS (PBST), and blocked with 10% FCS in PBST (20,24). The cells were stained with anti-PKC-α and -δ antibodies (Santa Cruz Biotechnology) as primary antibodies, washed, and incubated with FITC-conjugated secondary antibodies. To visualize the fluorescence, a Zeiss microscope was used.

DNA affinity precipitation assay

Two oligonucleotides containing biotin on the 5′ nucleotide of the sense strand were used in the assays. The sequences of these oligonucleotides are as follows: (i) COL-EBS oligo, 5′-GAAAGGGCGGGGGAGGGCGGGAGGATGCGGAGGGCGGAG, which corresponds to base pair −307 to −269 of

the human α2(I) collagen promoter, containing both an Ets-binding site (EBS) and GC-box, which can function as Sp1/3-binding sites (SBS); (ii) COL-EBS-M oligo, 5′-GAAAGGGCGGGGGAGGGCGGGAG**T**ATGCGGAGGGCGGAG, which has a mutated EBS of COL-EBS oligo. These oligonucleotides were annealed to their respective complementary oligonucleotides, and double-stranded oligonucleotides were gel-purified and used. Cell lysates were obtained using lysis buffer (25). Poly(dI–dC) competitor was incubated with the cell lysates, followed by incubation with each double-stranded oligonucleotide. After the incubation, streptavidin-agarose (Sigma) was added to the reaction and incubated. The protein–DNA–streptavidin–agarose complex was washed and loaded onto a SDS–PAGE gel. Detection of Sp1, Ets1 or Fli1 was performed with polyclonal IgG anti-Sp1, Ets1 or Fli1 antibody.

Plasmid construction

The generation of a −3500COL1A2/CAT construct consisting of the full-length human α2(I) collagen gene fragment linked to the chloramphenicol acetyltransferase (CAT) reporter gene and a series of 5′-deletions of the COL1A2/CAT construct were described previously (19,21,26). The substitution mutant plasmids in the CAGA motif which can function as a Smad-binding element (SBE) (located between bp −263 and −258) (SBE-mutated construct), the substitution mutants in the TCCTCC motifs (located between bp −128 and −123, or −164 and −159), and the substitution mutants in all three GC-boxes (located between bp −303 and −271) (SBS-mutated construct) of bp −353∼+58 deletion constructs of COL1A2/CAT were also described previously (19,26,27). Furthermore, point mutations changing GGAT to G**T**AT were introduced into the EBS (located between bp −285 and −282) of the bp −353∼+58 deletion construct of COL1A2/CAT (EBS-mutated construct) and the SBS-mutated construct (EBS/SBS-mutated construct) using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutation and deletion constructs were verified by sequencing.

Three copies of the 12 bp oligonucleotide [from bp −131 to −120 of the α2(I) collagen promoter] containing the TCCTCC motif were cloned in both orientations into the pBL-CAT5 vector carrying thymidine kinase gene promoter elements (21).

HA-tagged DN PKC-α, PKC-δ or PKC-ε, and HA-tagged wild-type (WT) PKC-α were kindly provided by Dr Jae-Won Soh (28). WT and DN PKC-δ were kindly provided by Dr Weiqun Li (29). DN PKC-ε was kindly provided by Dr Alex Toker (Deaconess Medical Center, Boston, MA) (30).

The expression vectors for Sp1 and Sp3 were kindly provided by Dr Guntram Suske (31,32), and the expression vectors for Ets1 and Fli1 were kindly provided by Dr Maria Trojanowska (33). Plasmids used in the transient transfection assays were purified twice on cesium chloride gradients as described previously (21). At least two different plasmid preparations were used for each experiment.

Transient transfection

Fibroblasts were grown to 50% confluence in 100 mm dishes in MEM with 10% FCS. The medium was replaced with serum-free medium, and fibroblasts were transfected with the α2(I) collagen promoter constructs, expression vectors or corresponding empty constructs, employing FuGENE6 as described previously (34). In order to correct minor variations in transfection efficiency, pSV-β-galactosidase vector (Promega, Madison, WI) was included in all transfections. After 48 h of incubation, cells were harvested in 0.25 M Tris–HCl, pH 8 and fractured by freeze-thawing. Extracts, normalized for protein content as measured with the Bio-Rad reagent, were incubated with butyl-CoA and [^{14}C]-chloramphenicol for 90 min at 37°C. Butylated chloramphenicol was extracted using an organic solvent (2:1 mixture of tetramethylpentadecane and xylene) and quantitated by scintillation counting. Each experiment was performed in duplicate.

Statistical analysis

Statistical analysis was carried out with the Mann-Whitney test for comparison of means. P values <0.05 were considered significant.

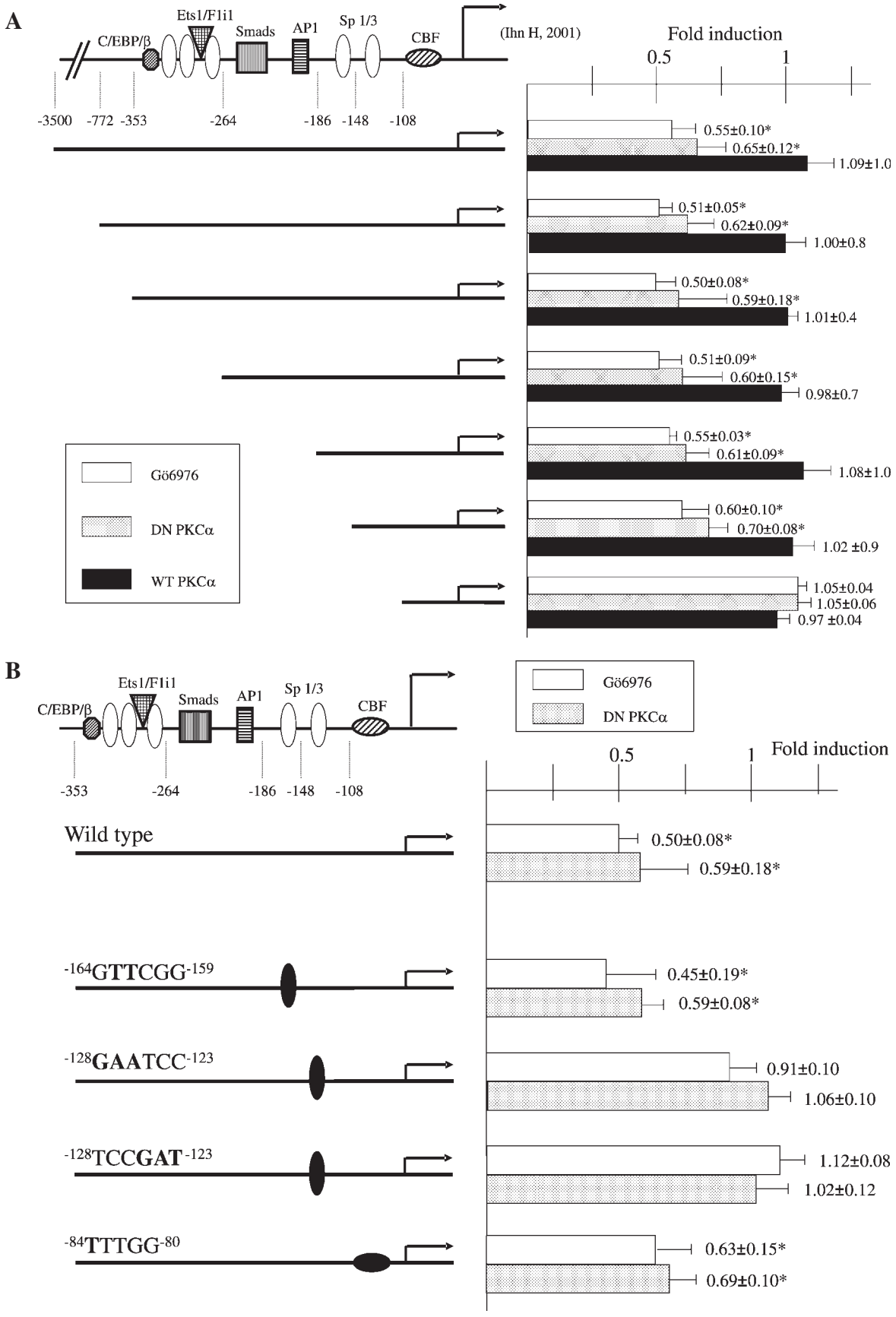
RESULTS

The effects of PKC inhibition on the expression of type I procollagen protein or the α2(I) collagen gene in normal dermal fibroblasts

First, we examined the effects of PKC inhibitors, calphostin C (whole PKC inhibitor), rottlerin and Gö6976 (specific PKC-α inhibitor), on the expression of type I procollagen in dermal fibroblasts by immunoblotting. As shown in Supplementary Figure 1A and B, two polypeptides, corresponding to the two chains of type I procollagen, were detected in the conditioned medium and cell lysates. It has been already shown that the altered ratio of the α1(I) to α2(I) chain is attributed to the difference in the immunoreactivity of anti-type I collagen antibody to the α1(I) and α2(I) chain (18). PKC inhibitors both decreased the secretion of type I procollagen into conditioned medium and reduced the deposition of type I procollagen in the cell lysates. To note, rottlerin had the greatest inhibitory effect (over 80% reduction), which was consistent with previous reports (15), whereas Gö6976 decreased the levels of type I procollagen modestly (almost 50% reduction). These results suggest that PKCs are involved in the basal expression of type I procollagen in dermal fibroblasts.

To determine whether the reduction of type I procollagen protein expression by these reagents was correlated with the corresponding mRNA levels, human dermal fibroblasts were incubated in the presence or absence of these inhibitors under the same conditions, and mRNA expression was analyzed by northern blotting. The α2(I) collagen mRNA level was significantly reduced after the stimulation with these reagents in comparison with the control level (Supplementary Figure 1C). However, the expression of GAPDH mRNA was not affected by these inhibitors, demonstrating that the indicated concentration of these inhibitors did not have generalized toxic effects. Thus, the effect of these inhibitors on the type I procollagen protein level paralleled that on the mRNA level.

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA.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Ets1/F1i1
C/EBP/β
Smads
AP1
Sp 1/3
CBF
(Ihn H, 2001)
Fold induction
0.5
1
-3500
-772
-353
-264
-186
-148
-108
0.55±0.10*
0.65±0.12*
1.09±1.0
0.51±0.05*
0.62±0.09*
1.00±0.8
0.50±0.08*
0.59±0.18*
1.01±0.4
0.51±0.09*
0.60±0.15*
0.98±0.7
0.55±0.03*
0.61±0.09*
1.08±1.0
0.60±0.10*
0.70±0.08*
1.02 ±0.9
1.05±0.04
1.05±0.06
0.97 ±0.04
Gö6976
DN PKCα
WT PKCα
B
Ets1/F1i1
C/EBP/β
Smads
AP1
Sp 1/3
CBF
Gö6976
DN PKCα
Fold induction
0.5
1
-353
-264
-186
-148
-108
Wild type
0.50±0.08*
0.59±0.18*
-164GTTCGG-159
0.45±0.19*
0.59±0.08*
-128GAATCC-123
0.91±0.10
1.06±0.10
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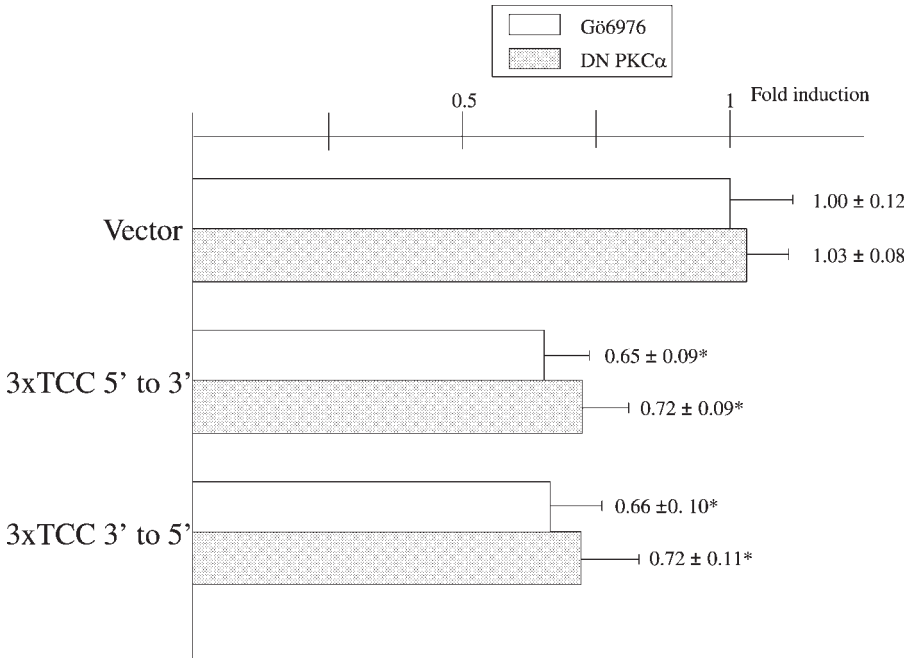
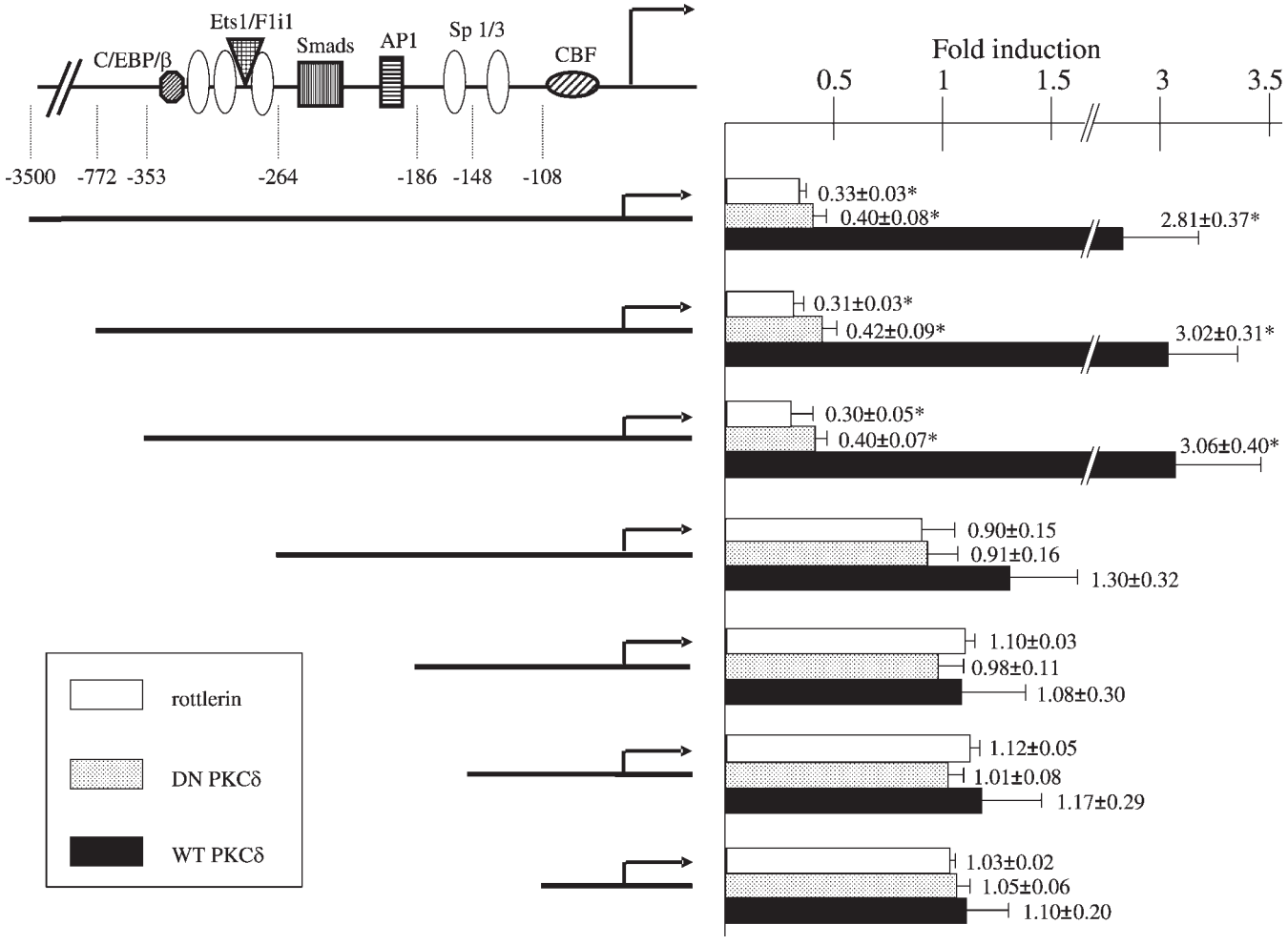
C


Gö6976
DN PKCα
0.5
1
Fold induction
Vector
1.00 ± 0.12
1.03 ± 0.08
3xTCC 5' to 3'
0.65 ± 0.09*
0.72 ± 0.09*
3xTCC 3' to 5'
0.66 ±0. 10*
0.72 ± 0.11*

D


Ets1/F1i1
C/EBP/β
Smads
AP1
Sp 1/3
CBF
-3500
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-353
-264
-186
-148
-108
Fold induction
0.5
1
1.5
3
3.5
0.33±0.03*
0.40±0.08*
2.81±0.37*
0.31±0.03*
0.42±0.09*
3.02±0.31*
0.30±0.05*
0.40±0.07*
3.06±0.40*
0.90±0.15
0.91±0.16
1.30±0.32
1.10±0.03
0.98±0.11
1.08±0.30
1.12±0.05
1.01±0.08
1.17±0.29
1.03±0.02
1.05±0.06
1.10±0.20
rottlerin
DN PKCδ
WT PKCδ

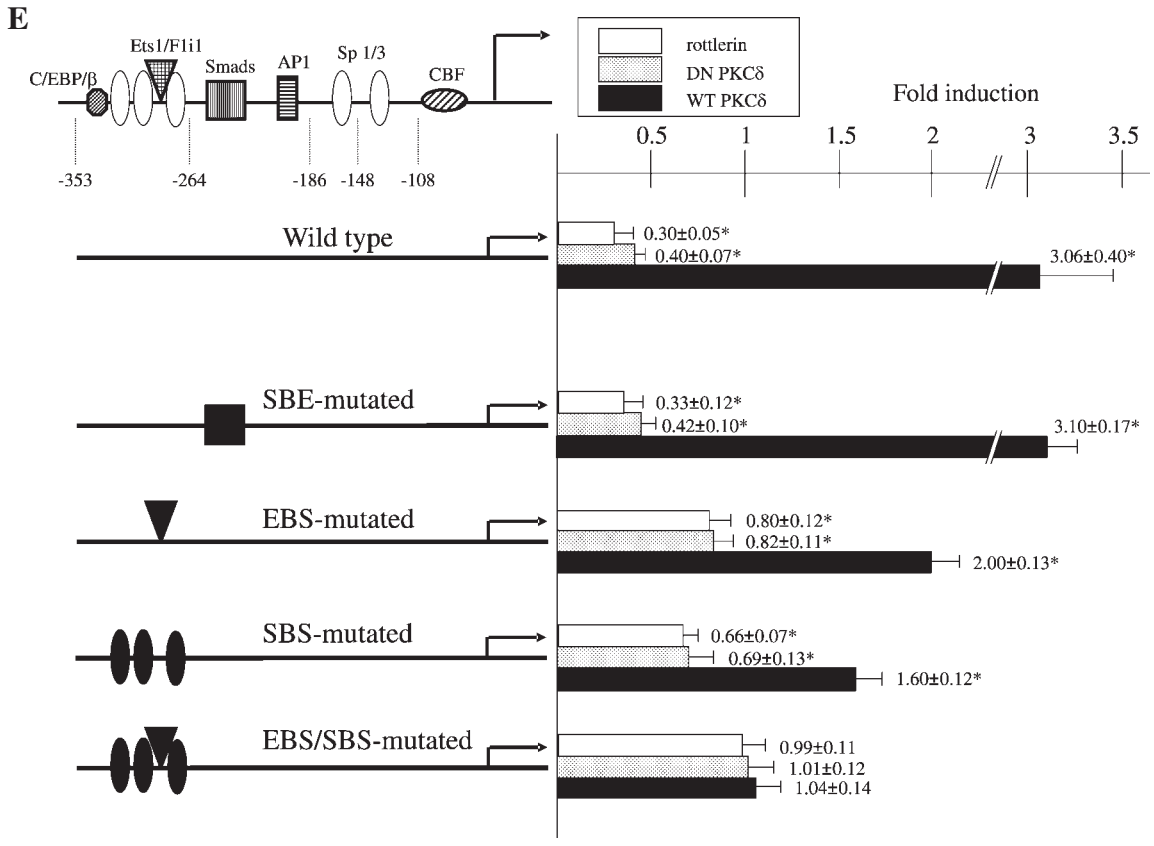


Figure 1. Identification of the α2(I) collagen promoter region mediating PKCs signaling in dermal fibroblasts. (**A**) The α2(I) collagen promoter deletion constructs were treated with Gö6976, or co-transfected with WT or DN PKC-α. The open bar graph represents fold-increase in the promoter activity on treatment with 2 μM of Gö6976 for 24 h relative to the promoter activity with vehicle, which was arbitrarily set at 1. The dotted or closed bar graphs represents fold-increase in the promoter activity on co-transfection with DN (dotted bar) or WT (closed bar) PKC-α relative to that with empty vector (0.5 μg), which was arbitrarily set at 1. The mean ± SE from four independent experiments is presented. *$P < 0.05$ as compared with the value in cells treated with vehicle or cells co-transfected with empty vector. (**B**) The diagram on the left indicates the mutant TCCTCC motif. Sequences with mutated nucleotides are indicated in boldface. Mutated plasmids were transfected in fibroblasts as described in Figure 1A. (**C**) The indicated plasmids (2 μg) were transfected into fibroblasts as described in Figure 1A. (**D**) The indicated α2(I) collagen promoter deletion constructs were transfected with 3 μM of rottlerin and 0.5 μg of DN PKC-δ or WT PKC-δ as described in Figure 1A. Open bar, cells treated with rottlerin; dotted bar, cells transfected with DN PKC-δ; and closed bar, cells transfected with WT PKC-δ. (**E**) Mutated plasmids were transfected with rottlerin, DN PKC-δ or WT PKC-δ as described in Figure 1D. SBE, Smad-binding element; EBS, Ets-binding site; and SBS, Sp1/3-binding site.

To establish whether the decrease in α2(I) collagen mRNA levels after the treatment with PKC inhibitors takes place at the transcriptional level or the posttranscriptional level, we wished to determine whether these reagents decreased the stability of the α2(I) collagen mRNA. Following the inhibition of transcription by the addition of actinomycin D, the loss of α2(I) collagen mRNA treated by the inhibitors was not significantly different from that observed in the untreated cells (Supplementary Figure 1D). The failure of these inhibitors to decrease the half-life of α2(I) collagen mRNA suggests that α2(I) collagen gene expression is regulated at the level of transcription by these inhibitors.

To confirm this, we determined the effects of these reagents on the α2(I) collagen promoter activity in dermal fibroblasts by conducting transient transfection assays using the full-length COL1A2/CAT construct. α2(I) collagen promoter activity was reduced by treatment with these inhibitors for 24 h to the same extent as the level of protein or mRNA (Supplementary Figure 1E). These results suggest that the basal activity of PKC is essential to maintain the basal transcriptional activity of the α2(I) collagen gene.

Several investigators doubt the specificity of Gö6976 and rottlerin toward PKC-α and -δ, respectively (35,36). Thus, we examined the effects of DN PKC-α or -δ on the expression of type I procollagen protein. The overexpression of these constructs also reduced the expression of type I procollagen protein in a dose-dependent manner (Supplementary Figure 1F), whereas DN PKC-ε had no effect, although HA-recombinant proteins were expressed at similar levels. These results confirm that basal type I procollagen protein or α2(I) collagen gene expression in normal fibroblasts is regulated by at least two signaling pathways, PKC-α and PKC-δ.

Functional analysis of the α2(I) collagen promoter by PKCs in normal fibroblasts

To identify potential regulatory elements of the human α2(I) collagen gene by Gö6976 or DN PKC-α, we performed transient transfection assays using a series of 5′-deletions of the α2(I) collagen promoter linked to the CAT reporter gene. Basal promoter activities of these constructs have been well examined previously (21).

The bp −148∼+58 construct and the longer constructs responded to Gö6976 or DN PKC-α, but the subsequent bp −108∼+58 construct showed little reactivity (Figure 1A). On the other hand, the overexpression of WT PKC-α did not affect any of these deletion constructs. The results indicate that the PKC-α inhibitor-response element is located between bp −148 and −108 in the α2(I) collagen promoter, and that the signaling activity of PKC-α is saturated in normal dermal fibroblasts.

This region contains the TCCTCC motif, which can function as SBS in the α2(I) collagen promoter (19) as well as integrin αv promoter (37). To determine whether this motif contributed to the PKC-α-mediated promoter activity, the effects of substitution mutations in the TCCTCC motif were investigated (Figure 1B). The α2(I) collagen promoter constructs carrying substitution mutations between bp −128 and −123 were unresponsive to Gö6976 treatment or co-transfection with DN PKC-α. None of the other substitution mutations in this promoter affected the effects of Gö6976 or DN PKC-α.

To further elucidate whether the TCCTCC sequence from the α2(I) collagen promoter mediated the effects of Gö6976 or DN PKC-α using a heterologous promoter, three copies of the 12 bp oligonucleotide (from bp 131 to 120) containing the TCCTCC motif were cloned in both orientations into the pBL-CAT5 vector carrying thymidine kinase gene promoter elements. The vector construct pBL-CAT5 alone was not affected by PKC-α inhibitors, whereas the insertion of 'TCC' oligonucleotides consistently resulted in a modest reduction in promoter activity by the inhibitors, independent of orientation (Figure 1C), which is similar to the previous report (21). These results suggest that PKC-α mediates the basal α2(I) collagen gene expression through the TCCTCC motif specifically in dermal fibroblasts.

Next, we examined the potential regulatory elements of the human α2(I) collagen gene by rottlerin or DN PKC-δ. Transient transfection assays using a series of 5′-deletions of the α2(I) collagen promoter revealed that the bp −353∼+58 construct and the longer constructs responded to rottlerin or DN PKC-δ, but these inhibitors did not affect the promoter activity of the subsequent bp −264∼+58 construct, indicating the PKC-δ-inhibitor-response element is located between bp −353 and −264 (Figure 1D). On the other hand, unlike WT PKC-α, the overexpression of WT PKC-δ-induced promoter activity, and their response element was also located between bp −353 and −264 in the α2(I) collagen promoter. This result confirms the PKC-δ responsive element, and indicates that the signaling activity of PKC-δ is not saturated in normal dermal fibroblasts.

This PKC-δ responsive element contains the EBS and three GC-boxes, which can function as the SBS (23,26, 38). Thus, the effects of substitution mutations at these binding sites were investigated (Figure 1E). Mutating all three SBS resulted in a significant reduction in reactivity to rottlerin, DN or WT PKC-δ, while mutating the EBS had a significant but weaker effect on the responsiveness. Mutating both the EBS and the SBS resulted in a complete abolishment of their effects. Mutating SBE did not affect the reactivity. Thus, the integrity of both the SBS and the EBS are thought to be critical for PKC-δ-mediated basal α2(I) collagen promoter activity.

Transcription factors involved in the α2(I) collagen gene's down-regulation by PKC inhibitors in normal fibroblasts

Our results suggest that Gö6976 or DN PKC-α down-regulates the α2(I) collagen gene expression via the SBS, whereas rottlerin or DN PKC-δ reduces it via the SBS and EBS. To further investigate the involvement of Sp1/3 in the effects of PKC inhibitors, we determined whether the amount of Sp1 or Sp3 was affected by PKC inhibitors. Immunoblotting revealed that the expression levels of these transcription factors in cell lysates were not altered by the treatment with Gö6976 or rottlerin (Figure 2A). Additionally, the phosphorylated levels of Sp1 were also unchanged (Figure 2B).

On the other hand, we determined whether forced overexpression of Sp1 or Sp3 could recover the PKC inhibitor-mediated reduction in type I procollagen protein. Twenty-four hours after transfection, the cells were treated with PKC inhibitors, and 72 h later the medium was collected. Immunoblotting revealed that transient transfection of Sp1 itself did not increase the expression of type I procollagen protein, but recovered the reduction by Gö6976 or rottlerin in a dose-dependent manner (Figure 2C). In particular, the effect of Gö6976 was completely recovered by Sp1 overexpression. The overexpression of Sp3 did not have such effects on type I procollagen protein expression (Figure 2D). These results confirm that Sp1 mediates the suppressive effects of PKC inhibitors without altering the expression or phosphorylation, and that PKC-α mediates basal collagen gene expression mainly via Sp1.

Next, we examined how the Ets family was correlated with the PKC-δ-mediated basal α2(I) collagen gene expression. In previous reports, both Ets1 and Fli1 were found to bind to the EBS between bp −285 and −282 of the α2(I) collagen promoter in a electrophoretic mobility shift assay using nuclear extracts from normal dermal fibroblasts. Furthermore, Ets1 and Fli1 had opposite effects on the α2(I) collagen gene expression: Fli1 inhibited the α2(I) collagen promoter activity by competing with Ets1 (23,38). Immunoblotting revealed that the amount of Ets1 in cell lysates was reduced and that of Fli1 was increased after the stimulation with rottlerin compared with the levels in control cells (Figure 2E). This indicated that rottlerin reduces Ets1 expression which has a positive effect on α2(I) collagen promoter activity, and induces expression of Fli1 which has a suppressive effect.

Additionally, to determine the binding activities of Ets1 and Fli1, we performed a DNA affinity precipitation assay using the COL-EBS oligo, containing the EBS and the SBS of the α2(I) promoter. As a negative control, we used the COL-EBS-M oligo, which has a mutated EBS of COL-EBS oligo. The results showed that only the COL-EBS oligo bound endogenous Fli1 strongly after rottlerin treatment for 3 h, whereas the binding of Ets1 to the EBS was decreased (Figure 2F). EBS-M oligo did not bind Ets1 or Fli1 even after the treatment with rottlerin. To note, the binding activity of Sp1 was unchanged by rottlerin in both EBS oligo and EBS-M oligo, however, it was decreased in EBS-M oligo. These results suggest that Ets1 or Fli1 binds to this site in a specific manner, and that the balance of Ets1 and Fli1 binding to the promoter by rottlerin mediates the suppression of α2(I) collagen promoter activity.

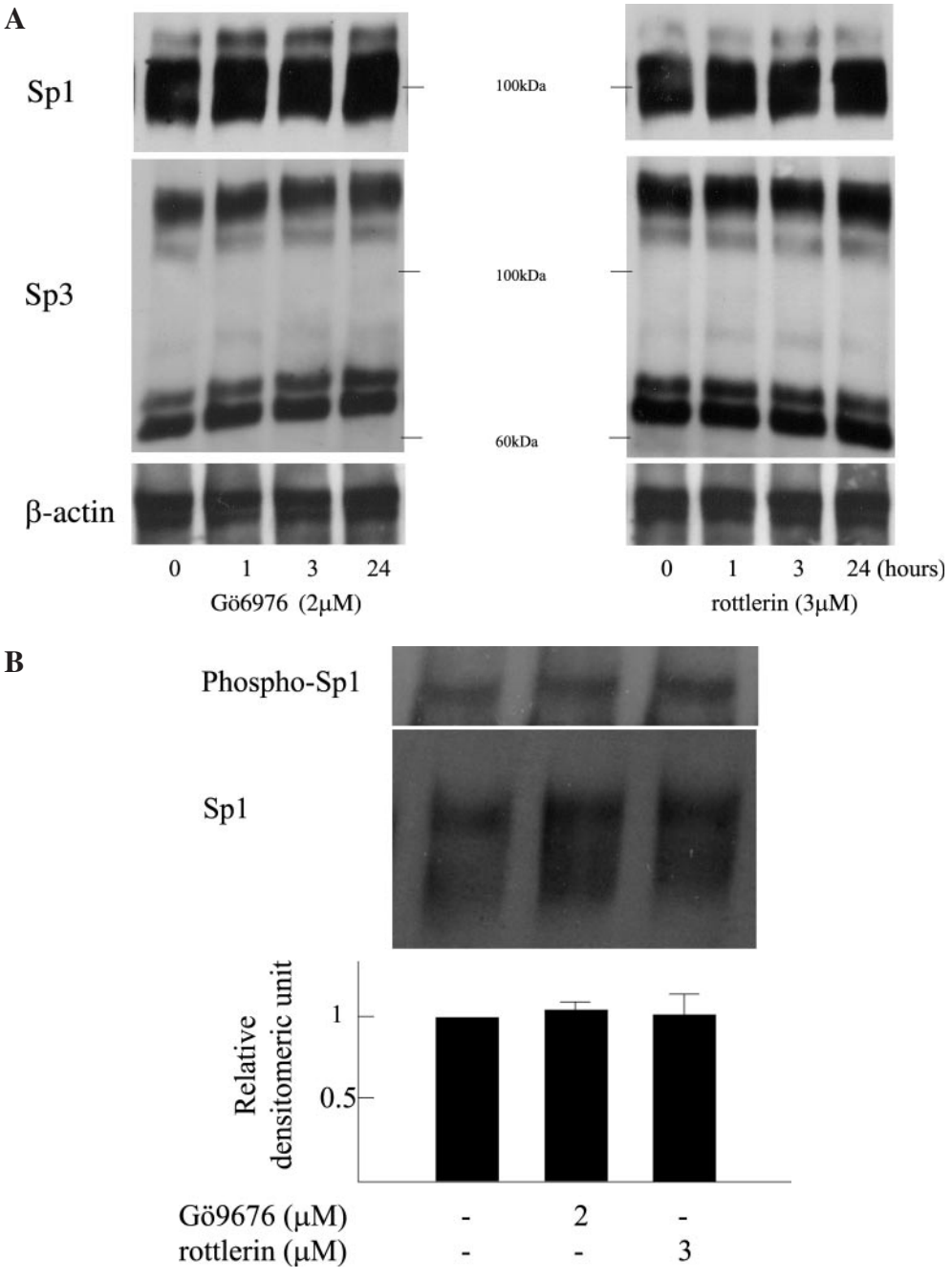
On the other hand, levels of threonine- or serine-phosphorylated Ets1 or Fli1 were unchanged by rottlerin (Figure 2G).

The effect of exogenous TGF-β1 on PKC signaling in normal fibroblasts

As shown above, the signaling activity of PKC-δ is not saturated in normal dermal fibroblasts. The balance of Ets1 and Fli1 mediated by the PKC-δ signaling pathway may be a novel mechanism regulating the basal α2(I) collagen expression. Thus, we determined whether PKC-δ or the balance of Ets1 and Fli1 is involved in the α2(I) collagen gene's up-regulation by exogenous stimuli, TGF-β1, as well as the basal expression.

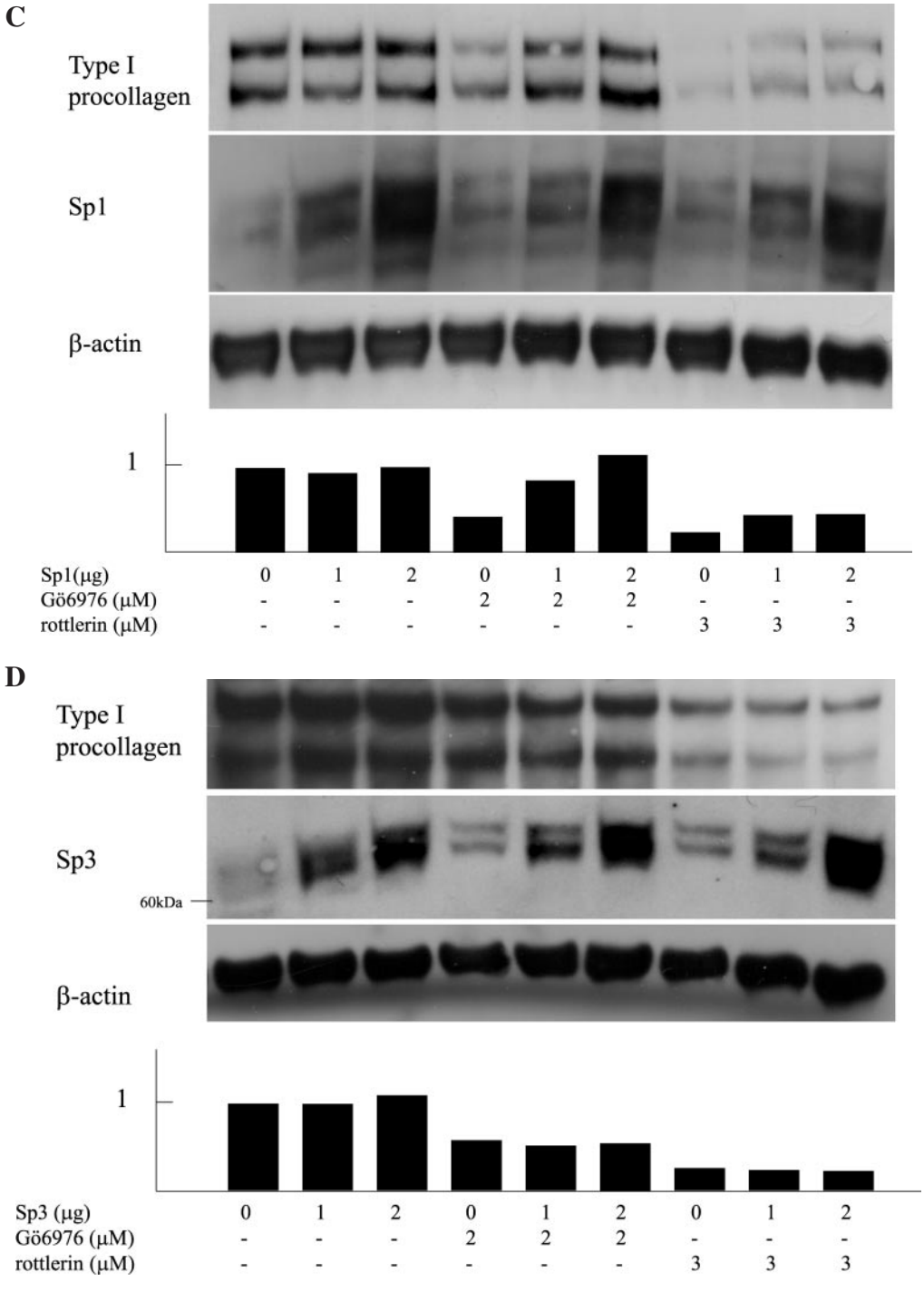
First, to examine the expression levels of PKC-α and -δ protein in the presence or absence of TGF-β1, we performed an immunoblot analysis using anti-PKC-α or -δ antibodies. Consistent with a previous report (39), TGF-β1 increased the expression of PKC-δ in dermal fibroblasts (Figure 3A). In contrast, PKC-α expression was not significantly affected by TGF-β1.

It is generally accepted that inactive PKC isoforms are located in the nucleus or cytoplasm, and translocated to the plasma membrane and/or cytoskeleton after activation (40,41). Immunofluorescent labeling revealed that the stimulation of dermal fibroblasts with TGF-β increased the size of each cell. Only PKC-δ was translocated to the plasma membrane and cytoskeleton in TGF-β1-treated dermal fibroblasts



(Figure 3B). These results indicate that PKC-δ expression is up-regulated and PKC-δ is activated by the treatment with TGF-β1.

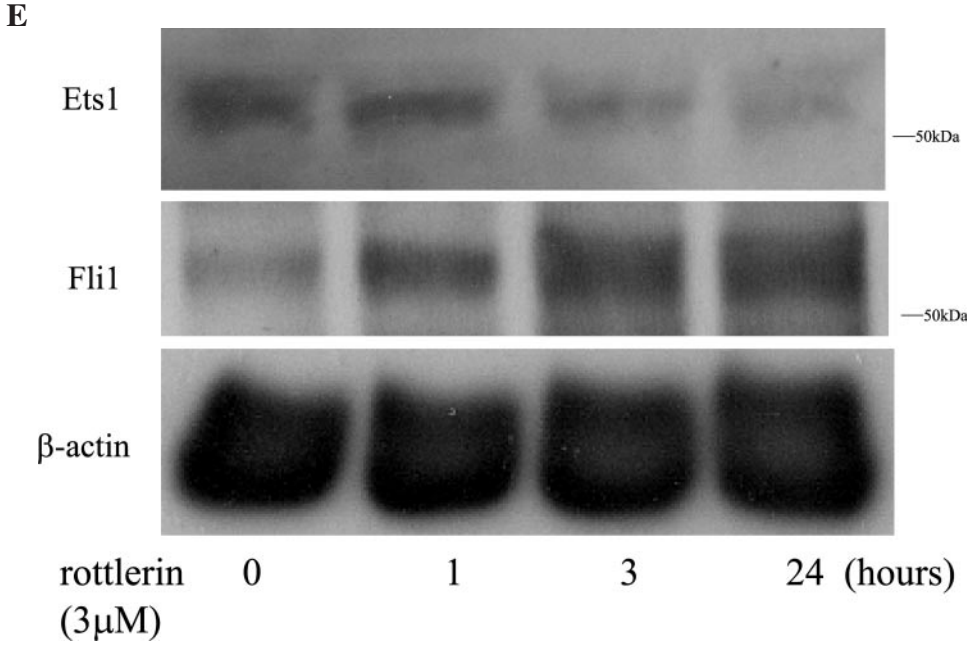
As mentioned above, the overexpression of WT PKC-δ increased the promoter activity in dermal fibroblasts, which is abolished by mutating both EBS and SBS between bp −353 and −264 of the promoter. Thus, we speculated that TGF-β1 affects the SBS and EBS via the PKC-δ signaling pathway. Because the SBS, the so-called GC-box, has been reported to be one of the TGF-β-response elements in the α2(I) collagen promoter (42,43), we then wished to confirm the involvement of the balance of Ets1 and Fli1 on EBS in the TGF-β1 signaling pathway. Thus, we determined the binding activities of Ets1 and Fli1, by conducting DNA affinity precipitation assays using the COL-EBS oligo. In fact, the addition of TGF-β1 increased the binding of Ets1 to the EBS and decreased that of Fli1 in normal fibroblasts (Figure 3C), although TGF-β1 was reported not to change Ets1 protein expression and to suppress Fli1 protein expression (23,44). These results indicate that the balance of Ets1 and Fli1 may also be involved in the TGF-β-mediated up-regulation as well as rottlerin-mediated down-regulation of the α2(I) collagen gene.

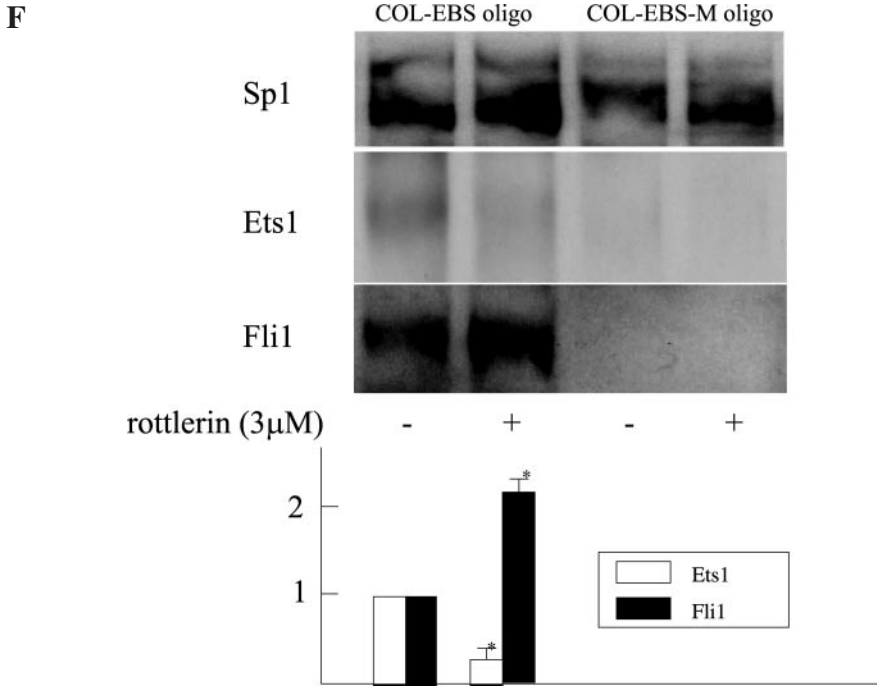


DISCUSSION

The PKC family of proteins is composed of at least 10 isozymes with diverse functions that are involved in numerous important cellular processes, mediating the specific activation of a variety of transcription factors, including AP-1 (45) and NF-κB (46). However, no previous reports have discussed the association between PKC and Sp1 or Ets family transcription factors.

Previously, Gö6976 was shown to have little or no effect on the α2(I) collagen mRNA expression or promoter activity in renal mesangial cells (47). However, there was a large difference in the dose of this reagent between our study and theirs. The expression of GAPDH mRNA was not affected in our study, indicating that the inhibitory effect of Gö6976 was not due to a generalized toxic effect. Furthermore, we confirmed the involvement of PKC-α in the regulation of α2(I) collagen gene expression by the overexpression of DN PKC-α. Our results suggest that Gö6976 as well as DN PKC-α downregulates α2(I) collagen gene expression via Sp1. However, Gö6976 did not alter the total amount of Sp1 or the level of phosphorylated Sp1. Although the mechanism by which Sp1 mediates the transcriptional regulation of the α2(I) collagen gene by PKC signaling is presently unknown, it is possible that PKCs affect the interaction of Sp1 with histones or other





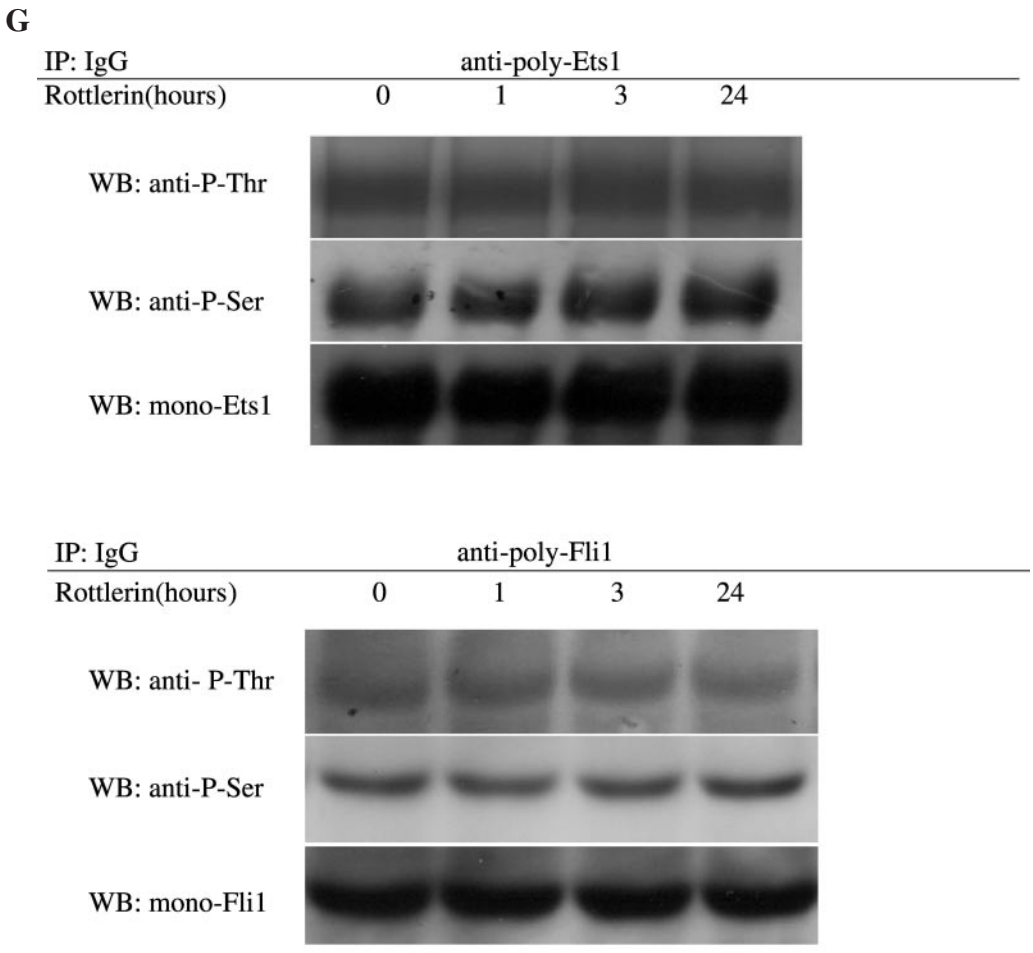


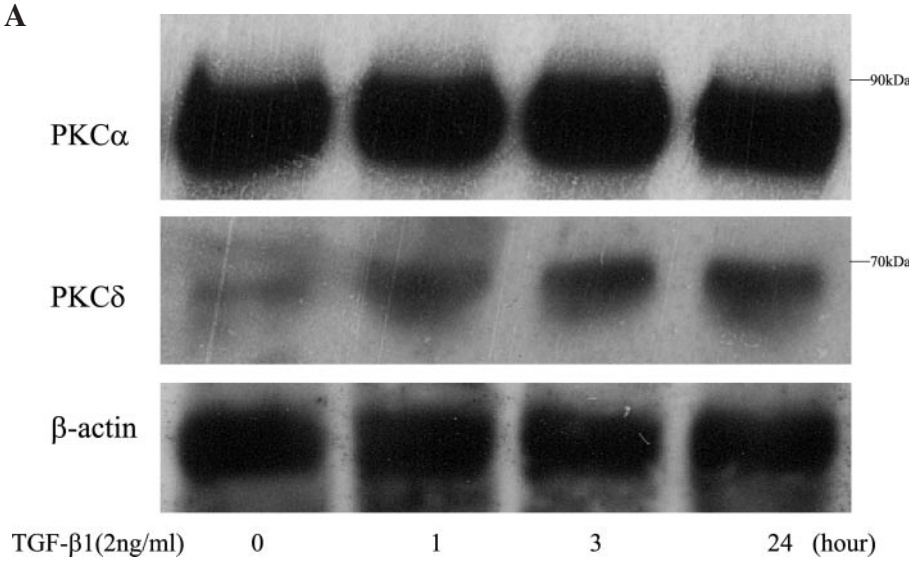
Figure 2. Participation of transcription factors in the α2(I) collagen gene's down-regulation by PKC inhibitors in normal fibroblasts. (**A**) To determine the amounts of Sp1 or Sp3 in cell lysates, fibroblasts were serum-starved for 24 h and treated with PKC inhibitors for the period indicated. Immunoblotting was performed using anti-Sp1 or Sp3 antibody. The same membrane was then stripped and reprobed with anti-β-actin antibody as a loading control. (**B**) Dermal fibroblasts were serum-starved for 24 h and treated with PKC inhibitors for 24 h. Cell lysates were immunoprecipitated with anti-Sp1 antibody to examine the phosphorylation levels of Sp1 as described in 'Materials and Methods'. One experiment representative of three independent experiments is shown. Phosphorylated Sp1 levels quantitated by scanning densitometry and corrected for the total level of Sp1 in the same samples are shown relative to the level in untreated cells (1.0). (**C** and **D**) For immunoblot analysis, the transient transfection of the Sp1 or Sp3 expression vector in normal dermal fibroblasts was performed as described in 'Materials and Methods'. Twenty-four hours after the transfection, cells were treated with the indicated dose of PKC inhibitors for 72 h. Conditioned media and cell lysates (normalized for protein concentrations as measured with the Bio-Rad reagent) were subjected to immunoblotting with anti-type I collagen antibody and antibody for HA or β-actin, respectively. Type I procollagen protein levels quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the level in untreated cells (1.0). (**E**) To determine the amounts of Ets1 or Fli1 in cell lysates, human dermal fibroblasts were serum-starved for 24 h and treated with rottlerin for the period indicated. Immunoblotting was performed using anti-Ets1 or Fli1 antibody. The same membrane was then stripped and reprobed with anti-β-actin antibody as a loading control. (**F**) Cell lysates were prepared from normal fibroblasts in the presence or absence of rottlerin for 12 h and incubated with the COL-EBS oligo or the COL-EBS-M oligo as described under 'Materials and Methods'. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Sp1, Ets1 or Fli1 was detected by immunoblotting. The levels of Ets1 (open bars) and Fli1 (closed bars) bound to COL-EBS oligo quantitated by scanning densitometry are shown relative to the level in untreated cells (1.0). The mean ± SE from four independent experiments is presented. $*P < 0.05$ as compared with the value in untreated cells. EBS, Ets-binding site. (**G**) Ets1 or Fli1 phosphorylation on threonine (top panel) or serine (middle panel) was determined in fibroblasts treated with rottlerin for the indicated periods of time. Ets1 or Fli1 levels were determined using monoclonal anti-Ets1 or Fli1 antibody on the same filter (bottom panel).

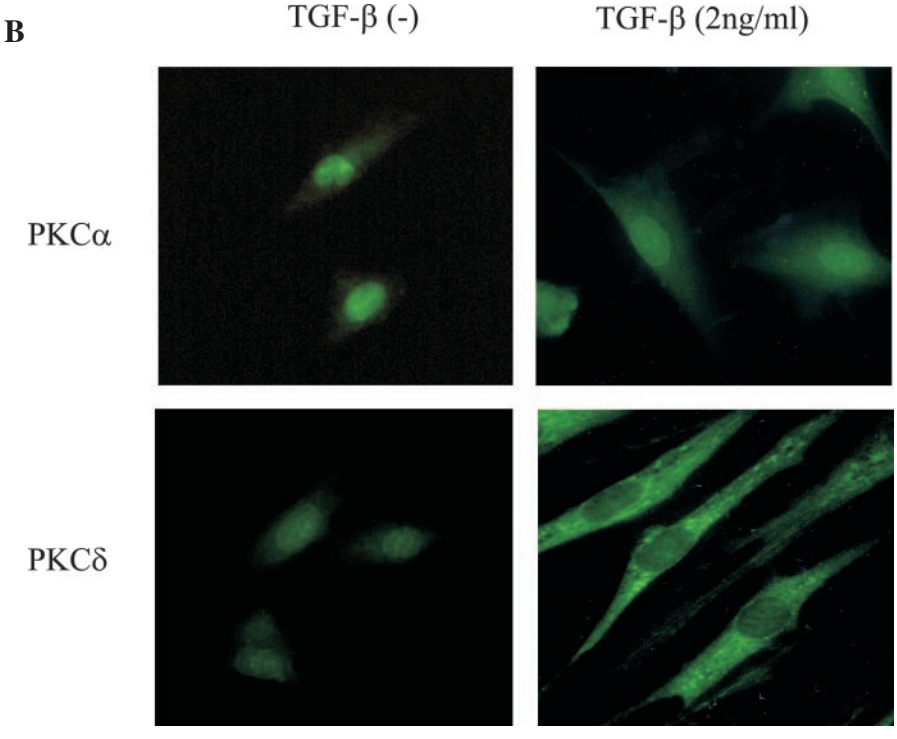
chromatin components (20). Such a mechanism has recently been described for the regulation of the mouse α2(I) collagen promoter by TGF-β/TNF-α, where these cytokine treatments affected the interaction of CTF/NF1 with histone H3 (48,49).

Our study indicated that PKC-δ mediates α2(I) collagen promoter activity via Sp1 and Ets1/Fli1 in normal fibroblasts. Synergism between Sp1 and Ets1 has been reported in parathyroid hormone-related protein promoters (50). Moreover, Sp1 and Ets1/Fli1 cooperation is implicated in the regulation of the α2(I) collagen promoter (23,38). Although rottlerin altered the Ets1/Fli1 ratio in the EBS of the α2(I) collagen promoter, this reagent did not change the levels of phosphorylated Ets1/Fli1 or binding activity of Sp1. Because their nucleotide-recognition elements lie in relatively close proximity, there may be synergism between Sp1 and Ets1/Fli1 in the regulation of this promoter through the modification of chromatin structure. This speculation is supported by the finding that the binding activity of Sp1 to GC-box is decreased by the mutation in the EBS, as shown in Figure 2F or Figure 3C.

Deletion analysis of the human α2(I) promoter identified active segments mediating basal promoter activity between bp −376 and −108 (51). Although PKC-α- and PKC-δ-response elements shown by our results were included in this region, these two signaling pathways acted on separate regions of the promoter. It is known that each member of the PKC family can have independent effects on a single gene regulation. Overexpression of PKC-α or -δ was reported to induce collagenase promoter activity through a 12-*O*-tetradecanoylphorbol-13-acetate-response element (52). On the other hand, PKC-α and PKC-θ are thought to have opposite effects on calcineurin-regulated retinoid X receptor transcription during T-cell activation (53). Furthermore, the response element of PKC-α differs from that of PKC-θ in the MDR1 promoter in human breast carcinoma cells (54). A similar phenomenon may occur in the regulation of α2(I) collagen gene expression.

We also confirmed the association between PKC-δ and TGF-β1 stimulation. TGF-β-induced-PKC-δ signaling affected the SBS and the Ets1/Fli1 ratio in the EBS leading to the activation of the collagen promoter. The GC-boxes (42,43), the AP-1 binding site (55) and the SBE (56) have been reported as TGF-β-response elements in the α2(I) collagen promoter, and are located between bp −353 and −186 in the promoter. Our results indicated that the EBS is also involved in the TGF-β response, which is located in the same region. We speculate that PKC-δ translocated to the plasma membrane and/or cytoskeleton that may regulate transcription of the α2(I) collagen gene via downstream signaling cascades





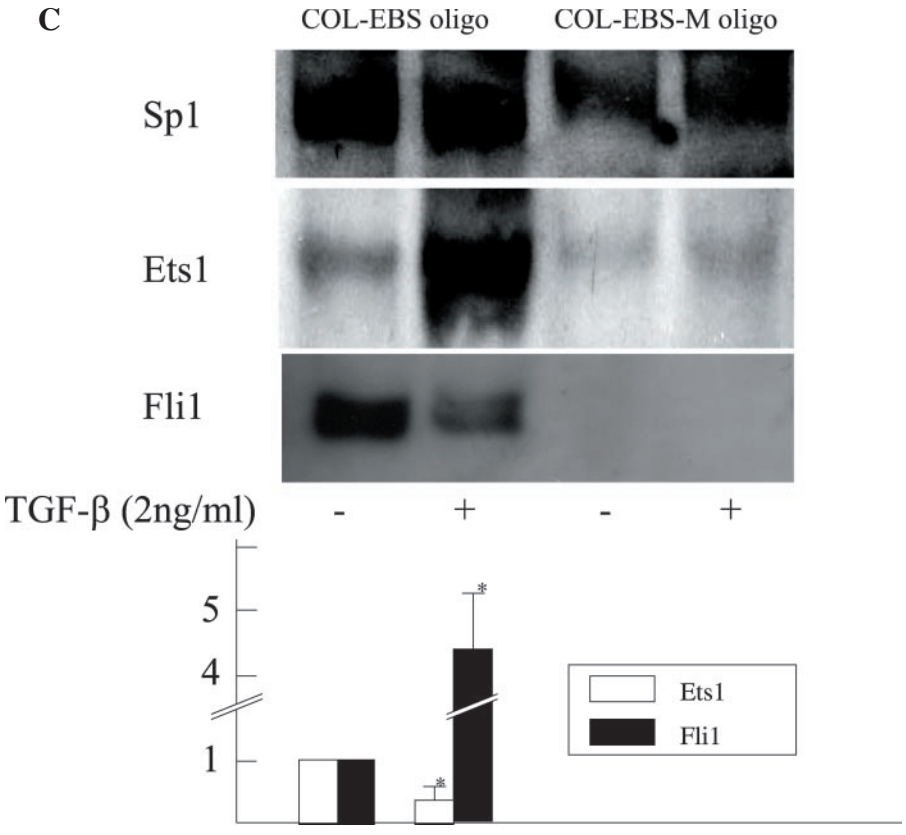


Figure 3. The effects of TGF-β1 on the expression of PKC-α and -δ in normal fibroblasts. (**A**) To determine the amounts of PKC-α and -δ in cell lysates, fibroblasts were serum-starved for 24 h and treated with TGF-β1 for the period indicated. Immunoblotting was performed using anti-PKC-α or -δ antibody. The same membrane was then stripped and reprobed with anti-β-actin antibody as a loading control. (**B**) The subcellular distribution of PKC-α and -δ was visualized using immunofluorescence. Fibroblasts were serum-starved for 24 h and incubated in the presence or absence of TGF-β1 for 1 h. Magnification: ×630. (**C**) Fibroblasts were serum-starved for 24 h and treated with TGF-β1 (2 ng/ml) for 12 h. Cell lysates were incubated with the COL-EBS oligo or COL-EBS-M oligo as described under 'Materials and Methods.' Proteins bound to each nucleotide were isolated with streptavidin-agarose beads, and Sp1, Ets1 or Fli1 was detected by immunoblotting. The levels of Ets1 (open bars) and Fli1 (closed bars) bound to COL-EBS oligo quantitated by scanning densitometry are shown relative to the level in untreated cells (1.0). EBS, Ets-binding site.

including Ras, Raf, MEK1/2 or ERK1/2. For example, it was recently reported that TGF-β activates ERK mitogen-activated protein kinase, which is implicated in transcriptional regulation by the Ets family, to enhance collagen expression in which PKC-δ may play some role (57,58). The balance of Ets1 and Fli1 may be a novel mechanism regulating α2(I) collagen expression. Taken together, our results suggest that PKC-δ is involved in the regulation of the α2(I) collagen gene in the presence or absence of TGF-β.

Kubo *et al*. reported that Fli1 protein expression is consistently down-regulated in scleroderma dermal fibroblasts (44). The up-regulation of PKC-δ and the down-regulation of Fli1 in scleroderma may be caused by the stimulation of TGF-β signaling. The inhibition of PKC-δ may be effective for the improvement of abnormal collagen overexpression. Further clarification of the role of PKC-δ in collagen expression could provide a novel strategy for the treatment of fibrotic reactions, including those occurring in scleroderma.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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