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The C-ABL Tyrosine Kinase Controls PKC δ Induced Fli1 Phosphorylation in Human Dermal Fibroblasts

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Abstract

Objective—We have previously demonstrated that in response to TGF β , Fli1 activity is repressed through a series of sequential posttranslational modifications, consisting of PKC δ induced Thr312 phosphorylation, acetylation by PCAF, and detachment from the collagen promoter. In this study, we further investigated the upstream events that lead to Fli1 phosphorylation in response to TGF β .

Methods—Fibroblasts were isolated from SSc and matched control patients. Western blot was used to analyze protein levels and quantitative real time RT-PCR was used to measure mRNA expression. Cells were transduced with constitutively active PKC δ adenovirus or transiently transfected with a bcr-abl overexpressing plasmid. Subcellular localization of PKC δ was examined by immunocytochemistry.

Results—Western blot analysis of cell lysates demonstrated that the levels of P-Fli1 (Thr321) were up-regulated in SSc fibroblasts, correlating with increased collagen type I and c-abl protein. Experiments using a constitutively activated form of c-abl, siRNA against c-abl and the specific tyrosine kinase inhibitor Imatinib, demonstrate that c-abl is required for the TGF β induced phosphorylation of Fli1. Additionally, we show that c-abl kinase activity is required for PKC δ nuclear localization.

Conclusions—Our results demonstrate that in SSc fibroblasts c-abl is an upstream regulator of the pro-fibrotic PKC δ /P-Fli1 pathway, via induction of PKC δ nuclear localization. Additionally, the finding that Fli1 is phosphorylated at higher levels in SSc fibroblasts supports the notion that the c-abl/PKC δ /P-Fli1 pathway is constitutively activated in these cells. Thus, blocking the TGF β /c-abl/PKC δ /P-Fli1 pathway could be an attractive alternative approach for scleroderma therapy.

Scleroderma (SSc) is an autoimmune connective tissue disorder characterized by vascular abnormalities and tissue fibrosis. In SSc, fibroblasts become activated and deposit large amounts of collagen and other extracellular matrix proteins. Although the precise pathogenetic mechanism remains unclear, it is generally considered that activation of TGF β pathways plays a major role in SSc fibrosis. TGF β acts through both canonical and non-canonical signal transduction pathways. Canonical TGF β signaling is initiated by ligand binding to the TBR1, which phosphorylates TBRI/Alk5 resulting in activation and nuclear translocation of the transcription factors Smad2/3. Activation of non-canonical signaling in SSc fibroblasts is characterized by elevated expression of Smad1, constitutive Smad1 phosphorylation, up-regulation of PKC δ and constitutive activation of downstream effectors of PI3K signaling such as Akt and c-abl [1].

PKC δ is a serine/threonine kinase implicated in a variety of cell functions, including cell cycle regulation and tissue remodeling [2,3]. Recent studies have revealed that PKC δ is involved in collagen gene regulation in normal and SSc fibroblasts, presumably via a Fli1 dependent mechanism [4,5].

Highly expressed in endothelial cells and cells of the hematopoietic system, Fli1 is a transcription factor belonging to the Ets family, which plays important roles in vasculogenesis, immune regulation, and malignant transformation and progression [6,7]. Although expressed at low levels in fibroblasts, Fli1 has a major antifibrotic role by controlling the expression of various extracellular matrix genes, including collagen type I [8], CCN2 [9], MMP1 [10] and tenascin-C [11]. Importantly, in a study from our laboratory, Fli1 was significantly under-expressed in endothelial cells and in dermal fibroblasts from SSc skin, and this correlated with elevated collagen deposition, thus suggesting a role for Fli1 in SSc fibrosis [12]. Under physiological conditions, Fli1 binds to the human $\alpha 2(I)$ collagen (COL1A2) promoter, repressing gene transcription [8]. We have recently described that in response to TGF β , Fli1 activity is repressed through a series of sequential posttranslational modifications, consisting of PKC δ induced Thr312 phosphorylation, acetylation by PCAF, and detachment from the collagen promoter, followed by Fli1 protein degradation [13]. In this study, we further investigated the upstream events that lead to Fli1 phosphorylation in response to TGF β .

Materials and Methods

Reagents

TGF- β 1 was obtained from Pepro Tech Inc (Rocky Hill, NJ) and the protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II were purchased from Calbiochem (San Diego, CA). Dulbecco's modified eagle medium (DMEM) and the antibiotic antimycotic solution (penicillin, streptomycin and amphotericin B) were obtained from Gibco BRL (Grand Island, NY) while fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Enhanced chemiluminescent (ECL) reagent and bovine serum albumin (BSA) protein assay reagent were obtained from (Thermo Scientific, Rockford, IL). TRIReagent was purchased from the Molecular Research Center. Primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL). The following antibodies were used: PKC δ (BD Transduction Laboratories), phospho-PKC δ (Cell Signaling Technology Inc., Danvers, MA), antitype I collagen (Southern Biotechnology, Birmingham, AL), β -actin antibody, clone AC-150 (Sigma), Egr1, lamin A/C, c-abl (Santa Cruz Biotechnology, Santa Cruz, CA) and P-c-abl (Tyr245) (Cell Signaling Technology Inc, Danvers, MA). The polyclonal rabbit anti-Fli1 and the phospho-Fli1 (Thr312)-specific antibodies were generated as described previously [13].

Cell culture

Human dermal fibroblast cultures were established as described previously [14] from the dorsal forearm of SSc patients and from age-, race-, and sex-matched healthy controls. Informed consent was obtained from all subjects, and the study was conducted in compliance with Institutional Review Board guidelines.

Western blots

Cells layers were harvested and lysed as described, either in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 15 mM NaF, 1 mM β -mercaptoethanol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) or in NE-Per nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. Protein concentration was

quantified using the BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Cell lysates were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, blocked for an hour in 2% milk at room temperature and immunoblotted with the indicated primary antibodies overnight in 2% milk at a dilution of 1:1000. Membranes were then washed and incubated for 1 h with the corresponding secondary antibody. Bands were detected using ECL.

Immunoprecipitation

In order to assess the endogenous levels of acetylated Fli1, 100 μ g of total cell lysates were subjected to immunoprecipitation using the Pierce Co-IP kit (Thermo Scientific, cat. No. 26149), according to the manufacturer's instructions. Briefly, 10 μ g of monoclonal mouse anti-Fli1 antibody were immobilized onto an agarose support, and then pre-cleared protein extracts were added overnight to the column. Next day the columns were washed and the remaining proteins were eluted, using the provided buffers. The precipitated proteins were subjected to immunoblotting using rabbit anti-acetylated lysine antibody (Cell Signaling Technology, cat. No. 9441).

RNA isolation, cDNA preparation, and quantitative real time RT-PCR

Total RNA was extracted using TRI Reagent and 1 μ g was converted to cDNA as previously described [15]. Quantitative real time RT-PCR was performed using SYBR Green mixture (Applied Biosystems, Carlsbad, CA) on a StepOnePlus Real-Time PCR system using 1 μ l of cDNA in triplicate with β 2-microglobulin as internal control. The following primers were used: β 2-microglobulin forward (GCC GTG TGA ACC ATG TGA CTT T), β 2-microglobulin reverse (CCA AAT GCG GCA TCT TCA AA); COL1A1 forward (CCA GAA GAA CTG GTA CAT CAG CA), COL1A1 reverse (CGC CAT ACT CGA ACT GGG AAT); COL1A2 forward (GAT GTT GAA CTT GTT GCT GAG G), COL1A2 reverse (TCT TTC CCC ATT CAT TTG TCT T); Fli1 forward (GGA TGG CAA GGA ACT GTG TAA), Fli1 reverse (GGT TGT ATA GGC CAG CAG); c-abl forward (CCC AAC CTT TTC GTT GCA CTG T), c-abl reverse (CGG CTC TCG GAG GAG ACG TAG A).

Immunocytochemistry

Dermal fibroblasts were grown on glass coverslips, serum starved and treated with 10 μ M Imatinib for 48 hours. Cells were then washed twice with 1 $\times$ PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature, followed by three washes in PBS. Cells were permeabilized with 0.25% Triton X-100 for 15 min followed by three washes in PBS and three hours blocking in 1% bovine serum albumin (BSA) in PBS at room temperature. Cells were incubated with Fli1 (rabbit) and PKC δ (mouse) primary antibodies (1:100 in 1% BSA in PBS) at 4°C overnight followed by three washes in PBS. The bound antibody was detected using anti-rabbit (Alexa Fluor 594, Invitrogen) and anti-mouse (Alexa Fluor 488, Invitrogen) secondary antibodies (1:200 each) overnight at 4°C. Coverslips were washed three times in PBS in the dark and then mounted on glass slides using Vectashield mounting medium with DAPI (Vector Laboratories Inc, Burlingame, CA). Slides were blinded, and ten random fields were examined using an Olympus microscope attached to a digital camera.

Inhibition of protein expression by small interfering RNA (siRNA)

For the inhibition of c-abl expression using siRNA oligos, dermal fibroblasts were grown to 80% confluence and transiently transfected with 50 μ M c-abl siRNA (Dharmacon, ABL1 ON-TARGETplus SMARTpool – a mixture of 4 siRNAs provided as a single reagent for enhanced potency and specificity), or the corresponding concentration of scrambled non-

silencing siRNA (Scrm, Qiagen) for 48 hours. Cells were then serum starved overnight and treated as indicated.

Adenoviral and plasmid constructs

Constitutively active PKC δ expressing adenovirus was a gift from Dr. Allan Samarel (Loyola University, Maywood, IL) and luciferase-expressing adenovirus was used as control. Expression of both PKC δ and luciferase was driven by the CMV promoter and constitutively active PKC δ was created by a point mutation (A159E) in the inhibitory pseudosubstrate domain [16]. Constitutively active c-abl in the form of the bcr-abl plasmid was obtained from Dr. John Varga (Northwestern University, Chicago, IL) and transient transfections were performed, according to manufacturer's instructions, using Attractene transfection reagent (Qiagen).

Statistical analysis

Statistical analysis was carried out using the Mann–Whitney test for comparison of means, and p-values less than 0.05 were considered to be statistically significant.

Results

Scleroderma dermal fibroblasts have increased levels of P-Fli1

To determine the phosphorylation status of Fli1 in SSc, five matched pairs of SSc and normal dermal fibroblasts were grown to confluence and serum starved overnight, then levels of P-Fli1 (Thr312) were measured via western blotting using a custom generated antibody. Figure 1a shows increased Thr312 phosphorylation of Fli1 in SSc fibroblasts in all the pairs tested, consistent with constitutive activation of the TGF β /PKC δ /P-Fli1 pathway in SSc. We also show that SSc fibroblasts have higher levels of acetylated Fli1 compared to normal control. This further confirms our initial finding showing that phosphorylation of Fli1 is a prerequisite for its subsequent acetylation in response to TGF β . Interestingly, high levels of P-Fli1 associated with an increase in both collagen and total and phosphorylated c-abl protein (Tyr245), thus suggesting a possible link between the c-abl and PKC δ /P-Fli1 pathway (Figure 1b). Confirming previously published data that showed down-regulation of Fli1 in SSc [12, 17], analysis of nuclear extracts revealed a decrease in total Fli1 protein levels in SSc fibroblasts (Figure 1b, right panel). Thus, the absolute levels of P-Fli1 were dramatically higher in SSc.

C-abl is required for the TGF β induced phosphorylation of Fli1 on Thr312

We next sought to determine whether c-abl is involved in TGF β induced Fli1 phosphorylation. To do this, we treated confluent serum starved normal dermal fibroblasts with 10 μ M of Imatinib, a c-abl tyrosine kinase inhibitor, for an hour prior to addition of 2.5ng/ml of TGF β . TGF β potently induced the phosphorylation of Fli1 on Thr312, thus confirming our previous report (Figure 2a). Additionally, in Imatinib pretreated cells, TGF β failed to induce Fli1 phosphorylation, suggesting that c-abl kinase activity is required for this event. To further validate the role of c-abl in Fli1 phosphorylation, we transiently transfected normal dermal fibroblasts with a plasmid overexpressing the bcr-abl fusion protein, which functions as a constitutively active form of c-abl. As shown in Figure 2b, bcr-abl induced Fli1 phosphorylation to similar levels as TGF β , thus supporting the role of c-abl in the phosphorylation of Fli1. Similar results were also obtained in NIH 293T cells transiently overexpressing Fli1 (data not shown). To further confirm the role of c-abl in TGF β -induced Fli1 phosphorylation, we depleted the endogenous levels of c-abl using siRNA oligos. The mRNA levels of c-abl were suppressed by up to 95% in siRNA treated cells (see Figure 3c). While TGF β induced Fli1 phosphorylation in scrambled, non-silencing

siRNA treated cells, fibroblasts with depleted c-abl failed to show an increase in the levels of P-Fli1 (Figure 2c). These data demonstrate that TGF β requires c-abl to mediate its biological effect on Fli1 phosphorylation in human dermal fibroblasts.

Inhibition of c-abl signaling increases total protein levels of Fli1 in cultured scleroderma dermal fibroblasts

According to our previous studies, TGF β decreases Fli1 protein stability [18]. We therefore investigated the effects of c-abl signaling on Fli1 protein levels. Two different approaches were used to down-regulate signaling through c-abl in cultured SSc fibroblasts: the pharmacologic inhibitor Imatinib and specific siRNA against c-abl. Both the c-abl siRNA and Imatinib treatment resulted in a significant increase (approximate 2 fold) in the total levels of Fli1, suggesting that c-abl is a negative regulator of Fli1 protein (Figure 3a and 3b). Because the observed increase in levels of Fli1 protein upon c-abl inhibition could be either due to increased transcription or to decreased protein turnover, we next examined the effect of c-abl siRNA on the mRNA levels of Fli1 by quantitative real time RT-PCR. Depletion of c-abl endogenous levels by more than 90% did not affect the levels of Fli1 mRNA, indicating that it is unlikely c-abl regulates Fli1 transcription (Figure 3c).

Constitutively active PKC δ overexpression partially rescues Imatinib mediated collagen down-regulation

To further examine the role of c-abl in PKC δ -induced Fli1 phosphorylation, we overexpressed a constitutively active form of PKC δ (CA-PKC δ) in normal dermal fibroblasts, in the presence or absence of Imatinib. Confirming previously published data [13] CA-PKC δ was sufficient to induce Fli1 phosphorylation even in the absence of TGF β stimulation (Figure 4a). Although c-abl was required for TGF β -induced Fli1 phosphorylation (Figure 2), Imatinib treatment had no effect on the levels of P-Fli1 in CA-PKC δ overexpressing cells, thus placing c-abl upstream of PKC δ in this process. Interestingly, in cells with constitutively active PKC δ /P-Fli1 signaling, Imatinib treatment only partially reduced collagen protein levels (by approximately 30%), as opposed to control cells (nearly 70%, figure 4a). Essentially the same results were obtained at the mRNA level (Figure 4c). However, Egr1 levels were completely depleted upon Imatinib treatment in both control and CA-PKC δ overexpressing cells. These results suggest that PKC δ /Fli1 and Egr1 are part of two parallel downstream pathways that independently regulate c-abl mediated basal collagen gene transcription. Together, these data suggest that Imatinib down-regulates collagen gene expression in part via a PKC δ /P-Fli1 dependent mechanism in human dermal fibroblasts.

Imatinib inhibits PKC δ nuclear localization in SSc dermal fibroblasts

Published data suggest that PKC δ undergoes nuclear cytoplasmic shuttling and that proper localization is important for its functions [2]. We next examined the effects of Imatinib treatment of SSc dermal fibroblasts on PKC δ subcellular localization by immunocytochemistry. All cells were positive for PKC δ as examined by counterstaining of the nuclei with DAPI. Confirming the work of Jimenez and colleagues [4], we found that in untreated cells, PKC δ preferentially accumulated in the nucleus of SSc dermal fibroblasts, although weak cytoplasmic staining was found in most cells (Figure 5, PKC δ). Almost all cells were also positive for Fli1 (approximately 85%, data not shown), although the intensity of the positive staining varied among cells. Similar to PKC δ and in agreement with published evidence [13], Fli1 had an exclusive nuclear localization (Figure 5, Fli1). Imatinib treatment resulted in translocation of PKC δ from the nuclear to the cytoplasmic compartment in the vast majority of cells examined, while it did not change the nuclear localization of Fli1. Since Fli1 phosphorylation requires nuclear localization of PKC δ , these

results suggest that Imatinib blocks Fli1 phosphorylation by inhibiting PKC δ nuclear localization.

Discussion

Major efforts have been made recently to identify non-canonical pathways downstream of TGF β that could be involved in the regulation of collagen gene expression in SSc, and in fibrotic diseases in general [1]. This study describes a novel TGF β /c-abl/PKC δ /P-Fli1 pathway that is a positive regulator of collagen synthesis and is constitutively activated in SSc fibroblasts. Under-expression of Fli1 has been previously reported in fibrotic conditions like SSc [12], cardiac fibrosis [19] and wound healing [20]. Recently, we have identified Fli1 phosphorylation (Thr312) as regulatory step that is a prerequisite for the subsequent acetylation and degradation of Fli1 in response to TGF β [13]. Analysis of cultured SSc fibroblasts showed up-regulation of P-Fli1 (Thr312), thus providing the first evidence that Fli1 is constitutively phosphorylated in SSc. This observation is particularly significant since Fli1 is already expressed at low levels in SSc fibroblasts [12], and posttranslational modification of Fli1 through phosphorylation further inhibits its activity [18]. Enhanced Fli1 phosphorylation correlated with an increase in Fli1 acetylation and collagen gene expression, suggesting that it could directly contribute to the pathogenesis of SSc fibrosis. Due to a partial loss of phenotype in SSc fibroblasts with serial passages, our further studies will be directed towards examining the expression of P-Fli1 in vivo in SSc biopsies. Further expanding our previous findings, this study also shows that the activation and nuclear translocation of PKC δ in response to TGF β is mediated through a c-abl dependent mechanism. C-abl is a non-receptor tyrosine kinase that is ubiquitously expressed and is implicated in a range of cellular functions [21]. The transforming variant bcr-abl has constitutively activated kinase activity, is involved in chronic myeloid leukemia (CML) and is targeted by the potent tyrosine kinase inhibitor Imatinib, which is currently the first line treatment for CML [22].

Imatinib also has antifibrotic effects by virtue of its ability to block the TGF β -induced activation of Smad1 and egr1 transcription factors [23,24]. In addition to its effects on egr1 and Smad1, we show here that Imatinib completely blocks TGF β -induced Fli1 phosphorylation on Thr312. Because Imatinib can target other tyrosine kinases, including PDGFR and c-kit, we utilized other approaches to demonstrate that Fli1 phosphorylation is dependent on c-abl activation. Our experiments using siRNA to deplete endogenous levels of c-abl showed that in the absence of this kinase, TGF β is unable to induce Fli1 phosphorylation. Further supporting the role of c-abl in this process, overexpression of a constitutively active form of c-abl resulted in a potent up-regulation of P-Fli1 levels. We have previously described that apart from leading to DNA dissociation, Fli1 phosphorylation also results in decreased protein stability. In the present study, we show that decreased expression of c-abl, or decreased c-abl kinase activity, can directly up-regulate total Fli1 protein levels in SSc fibroblasts. Several reports show that Imatinib can reduce collagen deposition in vitro and in animal models of tissue fibrosis. The value of Imatinib and other c-abl inhibitors in clinical fibrotic disease is still under investigation. Several case reports [25-27] have shown improved modified Rodnan skin score (MRSS) in SSc patients treated with Imatinib, and two patients have been described showing an Imatinib-responsive gene signature [27]. Open label clinical trials have suggested modest improvement in MRSS associated with Imatinib treatment [28]. However, controlled trials will be required to clearly define whether Imatinib is an active therapeutic for SSc.

Previous work has demonstrated that c-abl interacts with PKC δ in response to oxidative and genotoxic stress [29,30]. Thus, c-abl has been implicated both as a substrate for, and as an activator of the PKC δ kinase [2]. Our experiments using CA-PKC δ clearly show that c-abl

is a kinase upstream of PKC δ in the TGF β induced Fli1 phosphorylation. There is much confusion in the literature regarding the mechanism of PKC δ activation, various lines of evidence suggesting that distinct patterns of tyrosine phosphorylation could result in different and sometimes conflicting cellular outcomes, depending on the cell type, biological context and nature of the stimulus [2,3]. The PKC δ amino-acid sequence includes 19 tyrosine residues, out of which at least 9 have been implicated as phosphorylation sites. Further complicating these analyses, PKC δ can associate with different tyrosine kinases, including c-abl, Src, Lyn, Fyn, PYK2, LCK, and growth factor receptors, with variable outcomes [31]. Previous work has demonstrated that c-abl phosphorylates PKC δ in response to oxidative stress and ionizing radiation [29,32]. Importantly, c-abl-mediated tyrosine phosphorylation of PKC δ was necessary for its nuclear translocation and for induction of apoptosis in breast cancer cells [29]. We show here that in response to inhibition of c-abl kinase activity, PKC δ translocates from the nucleus – where it is preferentially localized in untreated SSc fibroblasts – to the cytoplasm. Thus, although the present results do not directly address the mechanism, the data clearly indicate that c-abl kinase activity is required for PKC δ localization to the nucleus, and subsequent Fli1 phosphorylation. Because other studies have shown that PKC δ can also be tyrosine phosphorylated by Src, resulting in its nuclear translocation [3], we sought to determine whether Src is involved in PKC δ mediated Fli1 phosphorylation. In cells treated with the selective Src family kinase inhibitor SU6656 and in cells overexpressing a dominant form of Src, basal levels of P-Fli1 were not decreased (data not shown), thus suggesting that Src is not involved in the phosphorylation of Fli1. PKC δ has been recognized as an important mediator of fibrosis in SSc, thus a potentially attractive target for therapeutic intervention. Recently, a selective pharmacological inhibitor of the PKC δ isoform - KAI-9803 - has been developed by KAI Pharmaceuticals. In a phase I clinical trial, KAI-9803 showed cardioprotective effects, being a safe adjunct to percutaneous coronary intervention in patients with acute ST-segment elevation myocardial infarction [33].

In conclusion, the present study shows that Fli1 is constitutively phosphorylated at higher levels in SSc, and c-abl is a pivotal activator of PKC δ that is essential for its nuclear localization and phosphorylation of Fli1. Thus, increasing or restoring expression of Fli1 by blocking the TGF β /c-abl/PKC δ /P-Fli1 pathway could represent an exciting potential therapeutic possibility against SSc fibrosis.

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Figure 1. P-Fli1 (Thr312) levels are up-regulated in cultured SSc dermal fibroblast

a) Scleroderma (S) and closely matched control dermal fibroblasts (N) were grown to confluence and then serum starved overnight. P-Fli1 and collagen levels were analyzed as described by western blot, and acetylated Fli1 (AcK-Fli1) was detected by immunoprecipitation using mouse anti-Fli1 antibody and by immunoblotting using rabbit anti-acetylated lysine antibody; bar graphs represent the densitometric analysis of western blots, with results expressed as arbitrary units $\pm$ standard deviation. (* $P < 0.05$ ** $P < 0.01$)

b) Representative western blot analysis of total cells lysates from matched scleroderma (S) and normal (N) pairs, showing association between up-regulation of collagen type I, c-abl and P-c-abl (Tyr245) protein levels and the increased Fli1 phosphorylation in SSc fibroblasts. Total Fli1 levels were analyzed by western blot in nuclear extracts and lamin A/C was used as control for equal loading.

Figure 2. TGFβ stimulates Fli1 phosphorylation through a c-abl dependent mechanism

a) Confluent dermal fibroblasts were serum starved overnight and treated with 10 μ M of Imatinib one hour prior to TGFβ stimulation, as indicated. Three hours after TGFβ addition cell layers were collected and the levels of phosphorylated Fli1 were analyzed by western blot.

b) Dermal fibroblasts were transiently transfected with bcr-abl plasmid or empty control, using Attractene transfection reagent. 48 hours later, control cells were stimulated with TGFβ for 3 hours, and then cells were harvested and analyzed for P-Fli1 levels.

c) Dermal fibroblasts were transiently transfected at 80% confluence with 50 nM of scrambled (Scr) or c-abl siRNA oligos, using HiPerFect transfection reagent. After 48 hours cells were serum starved overnight and then treated with TGFβ for 3 hours as shown. Cells were then collected and nuclear extracts were separated, and the levels of total and P-Fli1 were analyzed by western blot. Bar graphs represent the densitometric analysis of western blots. Lamin A/C was used as a control for equal loading.

Figure 3. Inhibition of c-abl in SSc fibroblasts results in up-regulation of Fli1 protein levels

a) SSc dermal fibroblasts were transiently transfected with 50 nM of scrambled (Scr) or c-abl siRNA oligos, using HiPerFect transfection reagent. Forty-eight hours later cells were serum starved overnight and then nuclear extracts were isolated and total Fli1 levels examined by western blot analysis.

b) Confluent SSc dermal fibroblasts were serum starved overnight and then treated with 10 μ M of Imatinib for 48 hours. The levels of total Fli1 protein were examined by western blot in nuclear extracts and densitometry results from three different experiments, in arbitrary units $\pm$ standard deviation, are shown in bar graphs. (* $P < 0.05$ ** $P < 0.01$)

c) Dermal fibroblasts were treated as described in a) and the mRNA levels of c-abl and Fli1 were analyzed using quantitative real time RT-PCR. Bar graphs represent the mean of three separate experiments in two different cell lines. (* $P < 0.05$ ** $P < 0.01$)

Figure 4. Overexpression of a constitutively activated form of PKCδ rescues Imatinib-mediated collagen protein down-regulation

a) Dermal fibroblasts were grown to confluence and then serum starved overnight. Cells were transduced with adenovirus carrying a constitutively active form of PKCδ (CA-PKCδ) or with control, luciferase adenovirus (C), in the presence or absence of 10 μM Imatinib (I). Forty-eight hours later, cells were scraped and protein expression was analyzed by western blot as previously described. A representative blot from three different experiments performed in two cells lines is shown.

b) Bar graphs representing the mean fold differences in arbitrary units (with standard deviation) of 3 independent experiments from western blots in a).

c) Dermal fibroblasts were treated as described in a) and the mRNA levels of COL1A1 and COL1A2 were analyzed using quantitative real time RT-PCR. Bar graphs represent the mean of three separate experiments in two different cell lines. (* $P < 0.05$ ** $P < 0.01$)

Figure 5. Imatinib inhibits the nuclear localization of PKCδ in SSc fibroblasts

a) Immunocytochemical co-localization of PKCδ and Fli1 in cultured SSc dermal fibroblasts was examined in the presence and absence of Imatinib. Cells were plated on coverslips and after overnight starvation were treated with 10 μ M of Imatinib for 48 hours. Immunocytochemical analyses for PKCδ (green) and total Fli1 (red) were performed as described in the Materials and Methods section. DAPI was used to counterstain the nuclei. The experiment was repeated in four SSc cell lines and representative images from two experiments are shown.

b) Western blot showing the effects of Imatinib on nuclear PKCδ in scleroderma fibroblasts. Lamin A/C was used as a control for equal loading.