Transcription Factor Fli1 Regulates Collagen Fibrillogenesis in Mouse Skin

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Biosynthesis of fibrillar collagen in the skin is precisely regulated to maintain proper tissue homeostasis; however, the molecular mechanisms involved in this process remain largely unknown. Transcription factor Fli1 has been shown to repress collagen synthesis in cultured dermal fibroblasts. This study investigated the role of Fli1 in regulation of collagen biosynthesis in mice skin in vivo using mice with the homozygous deletion of the C-terminal transcriptional activation (CTA) domain of the Fli1 gene (Fli1ΔCTA/ΔCTA). Skin analyses of the Fli1 mutant mice revealed a significant upregulation of fibrillar collagen genes at mRNA level, as well as increased collagen content as measured by acetic acid extraction and hydroxyproline assays. In addition, collagen fibrils contained ultrastructural abnormalities including immature thin fibrils and very thick irregularly shaped fibrils, which correlated with the reduced levels of decorin, fibromodulin, and lumican. Fibroblasts cultured from the skin of Fli1ΔCTA/ΔCTA mice maintained elevated synthesis of collagen mRNA and protein. Additional experiments in cultured fibroblasts have revealed that although Fli1 ΔCTA retains the ability to bind to the collagen promoter in vitro and in vivo, it no longer functions as transcriptional repressor. Together, these results establish Fli1 as a key regulator of the collagen homeostasis in the skin in vivo.

Fibril-forming collagens are the major structural components of the dermis responsible for its characteristic strength and resiliency. In the skin collagen fibrils are composed mainly of collagen type I and smaller amounts of collagen types III and V (11). Although collagen type V represents only a minor component of the fibril, it plays a key regulatory role in the process of fibrillogenesis (41). During physiologic remodeling, coordinate synthesis of specific collagen chains is tightly regulated (29), while during fibrosis the fibrillar collagens are produced at increased levels (37). The first critical step in the collagen biosynthetic pathway occurs at the level of transcription. In the past few years, a number of cis-regulatory elements and cognate transcription factors involved in type I collagen gene regulation at the basal level and in response to cytokines have been characterized in vitro studies (8, 13, 18, 36). Subsequently, several of these response elements, including Sp1 and CBF/nuclear factor 1 binding sites, have been validated in vivo in a transgenic mouse model (34). The in vivo studies have also underscored the complexity of the transcription regulation of the collagen gene, which involves interactions between transcription factor complexes at the proximal promoter and the far upstream enhancer (34).

Additional intracellular steps in collagen fibrillogenesis involve collagen folding and trimerization, which take place in the endoplasmic reticulum (3, 23). Prolyl-4 hydroxylase (P4H) and protein disulfide isomerase, which together form a P4H tetramer, catalyze formation of hydroxyproline, a critical step that facilitates folding and stabilization of the triple helix. The collagen-specific chaperone, HSP47, is also required for folding. Lysine residues are hydroxylated by a family of lysyl hydroxylases (also termed PLOD, for procollagen lysine 2-oxyglutarate 5-dioxygenase); this posttranslational modification contributes to formation of extracellular collagen cross-links. Further processing of collagen fibrils begins in the extracellular space with removal of N- and C-propeptides by C-proteinase (BMP-1) and N-proteinase (ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs). Removal of C-propeptide is essential to initiate self-assembly of collagen molecules into fibrils (35). Collagen fibrils increase in size through end-to-end fusion and lateral growth. Further strengthening of collagen fibrils requires inter- and intramolecular cross-links; this final step is catalyzed by lysyl oxidase. Other matrix molecules, including small leucine-rich proteoglycans (SLRPs), also regulate fibrillogenesis. Among SLRPs, decorin, fibromodulin, and lumican have been shown to bind to collagen fibrils and modulate their diameter and organization.

Fli1 is a member of the Ets family of transcription factors characterized by the presence of the evolutionary conserved DNA-binding (ETS) domain, which recognizes the purine-rich GGA(A/T) core sequence (27). Fli1 is preferentially expressed in hematopoietic cell lineages (38), and it is known to play a key role in megakaryocytic differentiation (12, 15). Fli1 is also involved in myelomonocytic, erythroid, and NK cell development (21). In addition, Fli1 is highly expressed in vascular endothelial cells, but its target genes and its role in the vasculature have not been fully characterized (10). Structurally, besides the ETS domain, Fli1 contains helix 1-loop-helix 2 domains in the 5′ region termed the N-terminal transcriptional activation domain and sequences that resemble turn-loop-turn secondary structure in the 3′ region termed the CTA (carboxy-terminal transcriptional activation) domain. Fli1 also contains a unique Fli1-specific region that contributes
to its role as transcriptional regulator (30). Depending on the promoter and the cellular context, Fli1 can function as a transcription activator or a repressor. There is evidence that the CTA domain mediates either the activator or repressor function of Fli1 (30). Initial deletion studies also indicated that this domain in EWS/Fli1 functioned as a transcriptional activator (26). However, subsequent studies using EWS/Fli1 fusion protein demonstrated that the CTA domain preferentially functions as a negative regulatory domain (1).

Studies in our laboratories using human dermal fibroblasts have implicated Fli1 in repression of type I collagen genes (6, 17). Our recent study has also demonstrated that reduction of Fli1 levels in dermal fibroblasts mimics, to a large extent, the transforming growth factor β-dependent profibrotic gene program, including increased synthesis of connective tissue growth factor (CCN2) and type I collagen and a reduction of MMP1 production (24). Since Fli1 protein is markedly downregulated in lesional fibroblasts of patients with scleroderma (17), the absence of Fli1 may contribute to the process of cutaneous fibrosis. Furthermore, a recent study has linked epigenetic mechanisms to the repression of the Fli1 gene in scleroderma skin in vivo (40). The goal of this study was to verify the role of the Fli1 gene in collagen biosynthesis in vivo using a mutant mouse model. The mice with targeted deletion of the Fli1 gene die at 11.5 days postcoitum. The early lethality is reported to be associated with vascular abnormalities. Heterozygous Fli1+/− mice are viable and fertile and lack any gross abnormalities (33). Because the early lethality of Fli1 null mice has limited functional studies in vivo, mice with targeted deletion of the Fli1 CTA domain (Fli1ΔCTA) were recently generated (42). We used this unique mouse model to investigate the role of Fli1 in regulation of connective tissue in the skin. Our results demonstrate that Fli1 and, in particular, its CTA are required for many aspects of collagen fibrillogenesis in the skin in vivo.

MATERIALS AND METHODS

Mice. We have generated a new mutant Fli1 line (termed Fli1ΔCTA/ΔCTA) by LoxP-Cre-mediated removal of the loxp-Banked (floxed) neomycin resistance gene present in the Fli1 locus of the original targeted mice (33). This floxed Neo cassette was inserted into the unique EcoRV site present in exon IX between the ETS DNA binding domain and the CTA domain. The resultant targeted allele also contains termination signals provided by the residual loxp sequence located between the DNA binding domain and the CTA domain. Thus, this new Fli1 allele expresses a truncated Fli1 protein (amino acids 1 to 384) that lacks the CTA domain. Fli1 heterozygous mice were crossed with CMV-Cre (where CMV allele expresses a truncated Fli1 protein (amino acids 1 to 384) that lacks the CTA domain. The resultant targeted allele is cytomegalovirus) transgenic mice that express Cre recombinase in all tissues, including germ cells. We obtained heterozygous Fli1+/− mice capable of germ line transmission of the truncated Fli1 gene. Mice were backcrossed to C57BL/6 and used for genotyping of mice, we used PCR to detect fragments of the CTA and littermate control male mice.

Genotyping. For genotyping of mice, we used PCR to detect fragments of the wild-type Fli1 and targeted Fli1 alleles. DNA was purified from tail clippings, and the PCR was as follows: 1 cycle at 94°C for 2 min followed by 35 cycles at 94°C for 2 min, 68°C for 1 min, and 72°C for 1 min. A 300-bp fragment indicates the presence of the wild-type allele. After Cre-mediated excision of the floxed neomycin cassette, the recombined allele (Fli1ΔCTA) retains a single loxp element as well as sequences derived from cloning, resulting in a 362-bp (309 plus 53 bp) fragment (24). The following primers were used: Fli1 Exon IX/Forward primer (nucleotides 1156 to 1180), GACAAACGGGAGTCTAAATGACG; and Fli1 Exon IX/Reverse primer (1441 to 1465), GGAGGATGGTGGAGAAACGGG. GACAAAG.

Quantitative real-time reverse transcription-PCR analysis. Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method (5). Real-time PCR assays were performed using a MyiQSingle-Color Real-Time PCR Detection system (Bio-Rad Cycler). Briefly, 5 μg of total RNA was reverse transcribed with random hexamers using a Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer’s protocol. The amplification mixture (10 μl) contained 0.125 μg of cDNA, 0.25 μM of each primer, and 5 μl of IQSYbr Green Supermix. Amplification was for 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. All samples were analyzed in parallel for β2-microglobulin expression as an internal control. The relative change in the levels of genes of interest was determined by the 2−ΔΔCt method. To compare the different samples in an experiment, RNA expression levels in samples were compared to expression of the control in each experiment. The primers for matrix-specific genes were previously described (19).

Extraction of collagen from skin by the acetic acid method with the addition of pepstatin. The acetic acid extraction of collagen was performed as previously described (19, 22). Skin punch (8 mm) samples were taken from the dorsi of each mouse. Next, skin pieces were minced and incubated in 10 volumes of phosphate-buffered saline overnight at 4°C with stirring. Tissue was harvested by centrifugation at 12,000 × g for 15 min and suspended in 10 volumes of cold 0.5 M acetic acid with or without the addition of pepstatin (1:10 ratio of pepstatin to tissue wet weight). Extraction was performed overnight at 4°C with stirring, and supernatant was dialyzed against 0.1 M acetic acid. Next, the dialysates with the addition of pepstatin were treated with pepstatin A (Sigma, Inc.), followed by lyophilization. Lyophilized proteins were resuspended in cold 0.1 M acetic acid and were tumbled for ~20 h. Equal aliquots from each sample were neutralized with 1 M Tris base, boiled in sample buffer with the addition of 2-mercaptoethanol, and resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. Collagen levels were quantitated using NIH Image densitometry software. Appropriate collagen bands were scanned using an Epson Perfection 4990 Photo Scanner. Band densities expressed arbitrary units were recorded.

Assessment of total collagen by hydroxyproline method. Measurement of hydroxyproline content in the skin was carried out as previously described (19). Briefly, hydrolyzed 8-mm skin punch samples were placed in 6 N HCl in sealed tubes and heated at 110°C for 3 h. After incubation the hydrolysates were transferred to 50-ml conical tubes containing 10 ml of distilled H2O and 2 ml of working buffer (0.5 M acetic acid, 0.7 M sodium acetate, 0.4 M sodium hydroxide, 1-propanol), and the solutions were vortexed. Adjustments to pH 7 to 8 were made using 4N NaOH and 6N HCl. Next, chloramine T was added to each sample, and samples were incubated at room temperature for 20 min, followed by addition of p-dimethyl-amino-benzaldehyde and a further incubation at 60°C for 15 min. The absorbance of the samples was measured in a spectrophotometer at 558 nm. The amount of collagen in each sample was calculated by comparison to a hydroxyproline standard curve and expressed as micrograms of hydroxyproline/milligram of tissue.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Sections 5 μm thick were mounted on APE5 (aminopropyltriethoxy silane solution) slides, deparaffinized in histoclear, rehydrated through a graded series of ethanol. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 30 min. Sections were then heated at 90°C for 45 min in Antigen Unmasking Solution (Vector Laboratories). To expose core proteins, sections were treated with appropriate enzymes (chondroitinase ABC for decorin; beta-endogalactosidase for lumican). The sections were then incubated overnight at room temperature with antibodies against decorin or lumican (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:100 in blocking buffer (1% rabbit serum), followed by incubation for 1 h with biotinylated rabbit anti-goat secondary antibody. The immunoreactivity was visualized with diaminobenzidine, and the sections were counterstained with hematoxylin.

EM. Sections of skin were generated from three wild-type (WT) and three Fli1ΔCTA/ΔCTA male mice at 3 month of age and were prepared for electron microscopy (EM) analyses in the EM Core Facility at the Medical University of South Carolina (MUSC). Briefly, after skin samples were collected from the back of control and Fli1ΔCTA/ΔCTA mice, the specimens were fixed in 4% paraformaldehyde–2% glutaraldehyde overnight followed by 1-h fixation in 1% OsO4 and embedding in Epon LX-112. Thin sections were stained with uranyl acetate and lead citrate and examined using a JEOL 1210 EM. Collagen fibril diameters were measured in scanned images generated from electron micrographs with NIH Image software. Collagen fibrils in at least three fields derived from sections of skin from each mouse were measured.

Cell culture. Mice skin tissue was rinsed extensively with antibiotic-antimycotic solution (Life Technologies). Tissue was dissociated enzymatically by 0.25% collagenase (Sigma) and 0.05% DNase (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) with 20% fetal bovine serum (FBS). Digested tissue was
placed in a 25-cm² flask in 5 ml of DMEM with 20% FBS and grown for 5 to 7 days. The resulting confluent culture was subsequently passed in DMEM with 10% FBS. Human dermal fibroblast culture was established from the foreskins of healthy newborns obtained from the delivery suites at the Medical University of South Carolina and in compliance with the Institutional Review Board. Cells between passage numbers 3 to 6 were used for experiments.

**Western blot analysis.** Cells were lysed in radioluminoprecipitation assay buffer. Total protein concentration was determined by biocinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts (25 to 50 µg) of total proteins per sample were separated via 6% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with polyclonal rabbit anti-mouse collagen type I antibody (Chemicon International, Inc.) at dilutions of 1:2,000 in 4% milk–Tris-buffered saline (TBS) overnight at 4°C. Where applicable, to expose core proteins, cell lysates were treated with appropriate enzymes (chondroitinase ABC for decorin, peptide N-glycosidase F for lumican, and β-endo-galactosidase for lumenic). Muscles were incubated with polyclonal antibodies against P4H, decorin, or lumican (Santa Cruz Biotechnology). The rabbit polyclonal antibody against Fli1 has been previously described (33); mouse monoclonal anti-Fli1 antibody was from BD Bioscience. After washes in Tween-TBS, membranes were probed with appropriate secondary antibody (Amersham Pharma- 
cisatawy, NJ) in 4% milk–TBS at a dilution of 1:500 for 1 h at room temperature. After washes in Tween-TBS, proteins were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

**Reporter gene assay.** pSg5-Fli1, pCTAP-Fli1, and −772 Col1A2/chromatin acetyltransferase (CAT) constructs were described previously (2). The Fli1 ΔCTA fragment was generated by PCR and subcloned into the pCTAP vector (Stratagene). Foreskin fibroblasts were grown to 50% confluence (Amersham Pharmacia Biotech, Piscataway, NJ) in 4% milk–TBS at a dilution of 1:500 for 1 h at room temperature. After washes in Tween-TBS, proteins were visualized using enhanced chemiluminescence reagents (Amersham).

**Promoter analysis and ChIP.** For prediction of putative transcription Ets factor binding sites, MatInspector professional software (Genomatix) was used as described in Quandt et al. (28). Genomic regions upstream of the transcription start sites for each gene of interest were extracted from the Cold Spring Harbor Laboratory Max mouse Promoter Database, version 2.3c (http://rulai.cshl.edu/cgi-bin/CShlpmd/mmpd.pl). Chromatin immunoprecipitation (ChIP) was performed as previously described (12). The following primers for PCR were used: Col1a1, 5′-CAGCCACACTCCAGTGACA-3′ (forward) and 5′-ACTCCCTGT CTCGCCGAGACT-3′ (reverse); Col1a2, 5′-TGCTGCTGTAACTCCTCAT GGU-3′ (forward) and 5′-CCTCCCTCTCACATCTCCT-3′ (reverse); Col3a1, 5′-CCACACATTTGACTTCACTCTTCTT-3′ (forward) and 5′-CCCTCCTGCTAT TCTCAGTA-3′ (reverse); Col5a1, 5′-CTTCAGAGCCGAGATCA-3′ (forward) and 5′-GTCCCTCTCCGGCTCTG-3′ (reverse); and Col5a2, 5′-GAG GGTGATGACAGACTCTTCTC-3′ (forward) and 5′-GCCCTGACTGACTAG AGTTT-3′ (reverse). The following cycling conditions were used: 94°C for 5 min; 36 cycles of 94°C for 30 s, 54°C for 60 s, and 72°C for 90 s; 72°C for 7 min; and 4°C to ∞. PCR products were separated on 2% agarose gel and stained with ethidium bromide.

**DNA affinity precipitation assay.** The biotin-labeled COILIA2 oligonucleotide (5′-GAAAAAGCGGGGGAGGCGGAGGATCGGGAGGGGAGG-3′), which corresponds to base pair −307 to −269 of the human α2(I) collagen promoter, containing both an Ets-binding site and GC box was used. After annealing to complementary oligonucleotide, double-stranded oligonucleotides were gel purified and used. Whole-cell extracts (500 µg) prepared from 293T cells were incubated for 10 min at 4°C with gel shift binding buffer (10 mM Tris-HCl [pH 8.0], 40 mM KCl, 1 mM dithiothreitol, 6% glycerol, 0.05% NP-40, and 20% polyethylene glycol) in a final volume of 1 ml. Precipitation was performed by adding streptavidin-coupled agarose beads and incubating the mixture for 30 min with gentle rocking at 4°C. After centrifugation, the supernatant was incubated with 500 pmol of each double-stranded oligonucleotide overnight at 4°C with gentle rocking. Then 65 µl of streptavidin-coated agarose beads was added, followed by a further 2-h incubation at 4°C. The protein-DNA-streptavidin agarose complex was washed twice with wash buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl), twice with gel shift binding buffer, and once with phosphate-buffered saline. Precipitates were subjected to immunoblotting using the antibodies indicated in the figures.

**Statistical analysis.** The Student’s t test analysis using GraphPad InStat Statistics software (version 1.12) was performed to determine statistical significance. Values of less than or equal to 0.05 were considered statistically significant.

**RESULTS**

The Fli1 CTA domain mediates repression of collagen genes in vivo. Previous studies have indicated that the CTA domain of Fli1 is involved in transcriptional modulation of a subset of target genes (1). To determine whether this domain is involved in collagen gene regulation, mice with the homozygous deletion of the COOH terminus of Fli1 gene (Fli1ΔCTA/ΔCTA) were utilized in this study. The absence of the CTA domain in mutant Fli1 mice was confirmed by PCR using primers specific for this domain (Fig. 1A). The levels of Fli1 mRNA in skin tissues were increased by ~70% in Fli1ΔCTA/ΔCTA mice compared to the respective WT controls as measured by quantitative real-time PCR (Fig. 1B). Furthermore, protein levels of truncated Fli1 were markedly increased in fibroblasts isolated from Fli1ΔCTA/ΔCTA mice (Fig. 1C). Elevated expression of Fli1 was also observed in the skin, lungs, and heart tissues of Fli1ΔCTA/ΔCTA mice (data not shown). These data suggest that the absence of the CTA domain of Fli1 may interfere with the autoregulation at the transcriptional level, consistent with recent studies that demonstrated involvement of Fli1 in regulating its own expression in lymphocytes (25). We next quantified procollagen type I, III, and V mRNAs using quantitative PCR in the skin tissues of WT and Fli1ΔCTA/ΔCTA mice. Absence of the CTA domain had a stimulatory effect on collagen mRNA levels in the skin. Col1a1 increased (6.2 ± 0.6)-fold (P < 0.04), Col1a2 increased (3.2 ± 1.2)-fold, Col3a1 increased (4.4 ± 0.6)-fold (P < 0.05), Col5a1 increased (3.7 ± 0.7)-fold (P < 0.05), and Col5a2 increased (6.0 ± 0.8)-fold (P < 0.01) (Fig. 1D). Likewise, Col1a1 mRNA increased (5.4 ± 3.4)-fold (P < 0.05; n = 4) in the heterozygous Fli1ΔCTA mice, suggesting that the truncated Fli1 protein acts as a dominant negative form.

To further investigate the effects of Fli1 ΔCTA on collagen species in the skin, collagen was extracted with 0.5 M acetic acid with the addition of pepsin (22). Since both soluble and insoluble collagens are efficiently extracted by this method, it provides relatively accurate assessment of the total collagen content. The patterns of collagen bands were similar in all samples, suggesting no qualitative differences in collagen composition (Fig. 1E). Significantly more collagen was extracted from the skin of Fli1ΔCTA/ΔCTA mice compared to controls. Furthermore, hydroxyproline content was also significantly increased in the Fli1ΔCTA/ΔCTA mice compared to controls (37.4 ± 5.0 µg of hydroxyproline/mg of tissue versus 24.8 ± 4.7; P < 0.03; n = 3). Together, these observations indicate that the CTA domain of Fli1 plays a critical role in repressing fibrillar collagen gene expression in mouse skin. However, the increase of collagen mRNAs in the skin of mutant mice was more pronounced than the corresponding increase in deposition of collagen protein, suggesting that transcriptional derepression of collagen genes is not sufficient to drive the process of fibrillogenesis in vivo.

Selected SLRPs are downregulated in the skin of Fli1ΔCTA/ΔCTA mice. Previous studies have implicated SLRPs in regulation of collagen fibrilogenesis (4, 31). Therefore, we next investigated the expression of decorin, fibromodulin, and lumican in skin of Fli1ΔCTA/ΔCTA and control WT mice. The mRNA levels of these molecules were measured by quantitative PCR. In Fli1ΔCTA/ΔCTA mice, the mRNA levels of decorin decreased 65% (P < 0.05), and lumican decreased 63% (P < 0.04),
FIG. 1. Fli1 ΔCTA modulates mRNA and protein expression of fibrillar collagens in mice skin. (A) Diagram of wild-type Fli1 and Fli1 ΔCTA constructs (left). Primers A and B are located in the N terminus, while primers C and D are located in Ets domain and CTA domain, respectively. Reverse transcription-PCR analysis was performed of RNAs isolated from cultured dermal fibroblasts derived from the back skin of 12-week-old WT and Fli1 ΔCTA ΔCTA mice. Two distinct primer sets shown in left panel were used. The arrow points to the expected size of the product. (B and D) Quantitative real-time PCR analysis of gene expression of Fli1 (B) and fibrillar collagens (D) in WT (white bars) and Fli1 ΔCTA ΔCTA (black bars) mice. Total RNA was isolated from skin punches. Quantitative real-time PCR analysis was performed with Sybr green and β2-microglobin as an internal control. The expression level of each gene in control mice was set at 1. Data were obtained from duplicate samples from at least four mice in each group. Values are presented as means ± standard deviations. *, statistically significant value. (C) The expression levels of Fli1 protein were determined in cultured dermal fibroblasts by immunoblotting following immunoprecipitation (IP). An equal amount of cell extracts was used for immunoprecipitation with mouse monoclonal anti-Fli1 antibody using mouse immunoglobulin G-specific immunoprecipitation matrix. Precipitated proteins were subjected to immunoblotting with mouse monoclonal anti-Fli1 antibody and ExactaCruz E Western blot reagent (horseradish peroxidase-conjugated secondary antibody). (E) Pepsin-soluble collagen was prepared as described in Materials and Methods. Equal aliquots from each sample were resolved by 6% SDS-PAGE and stained with Coomassie blue (left panel). Arrows indicate collagen α1(I) and α2(I) subunits. β-Components represent cross-linked α-chain dimers. Graphical representation of collagen levels was quantitated using NIH Image densitometry software. Values are the means ± standard deviations from at least 3 mice in each group. *, statistically significant values.
whereas fibromodulin mRNA levels were only marginally decreased (33%) (Fig. 2A). We have also analyzed the expression levels of two of the enzymes involved in collagen cross-linking. Plod2 mRNA levels were significantly increased in Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice, while the Lox levels were unchanged (Fig. 2A). There were large variations in the expression levels of Plod2 in both control and Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice. The protein expression levels of decorin, lumican, and fibromodulin were assessed by immunostaining of skin sections from the Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ and control mice. The skin sections were pretreated with the appropriate enzyme (chondroitinase ABC for decorin and β-endogalactosidase for lumican).

**Fig. 2.** Expression of matrix-related genes differs in Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice. (A) Quantitative PCR analysis of gene expression of decorin (Dcn), fibromodulin (Fmod), lumican (Lum), PLOD2, and lysyl oxidase (Lox) in control (white bars) and Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ (black bars) mice. The expression level of each gene in control mice was set at 100. Data were obtained from duplicate samples from at least 5 mice in each group. Values are presented as means ± standard deviations. *$P<0.05$, statistically significant values. (B) Immunohistochemistry was performed on paraffin-embedded, formalin-fixed tissue from control and Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice (original magnification, ×200; scale bar, 50 μm). To expose core proteins, sections were treated with the appropriate enzyme (chondroitinase ABC for decorin and β-endogalactosidase for lumican).

besides interstitial collagens, Fli1 also regulates expression of selected SLRPs.

**Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice have abnormal collagen fibrils.** Reduced expression levels of SLRPs in Fli1$^{\Delta}\text{CTA}$ mice suggested the possibility that collagen fibrillogenesis may be defective in these mice. Therefore, distribution of collagen fibrils was analyzed by transmission EM. In contrast to WT mice, collagen fibrils with irregular sizes and shapes were present in the skin of the Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice (Fig. 3). There was an overall increase of fibril diameter in Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice. In addition to fibrils with normal diameters, there were much thicker, irregularly shaped fibrils. Mean fibril diameter was 118.6 ± 39.2 nm in Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mice and 92.7 ± 34.5 nm in the WT ($P<0.05$). Furthermore, Fli1$^{\Delta}\text{CTA}/\Delta\text{CTA}$ mouse skin showed a wide range of fibril diameters in cross-section (22 to 302 nm in WT and 15 to 511 nm in mutants), indicating the increase of immature thin collagen fibrils as well as thick fibrils.
Loss of repressor function of Fli1 ΔCTA is maintained in cultured fibroblasts. To further analyze the functional role of the CTA domain of Fli1, cell cultures were established from the skin sections of WT and Fli1ΔCTA/ΔCTA mice. Consistent with in vivo data, fibroblasts from Fli1ΔCTA/ΔCTA mice showed a significant increase in mRNA levels of Col1a1 (5-fold), Col1a2 (1.2-fold), Col3a1 (4.5-fold), Col5a1 (3.3-fold), Col5a2 (4.3-fold), and Col5a2 (4.3-fold) compared to WT fibroblasts. These data indicate that Fli1 is directly responsible for the repression of fibrillar collagens in vivo.

We have previously demonstrated that Col1a1 mRNA and protein levels are inversely proportional to the Fli1 expression levels in mouse embryonic fibroblasts (MEFs) isolated from WT and Fli1ΔCTA/ΔCTA mice. Consistent with this process, protein levels were also markedly elevated in Fli1ΔCTA/ΔCTA cells, thus verifying the specificity of the antibody. To determine if the increase of DNA binding of Fli1 ΔCTA was also increased in comparison to WT Fli1 protein, we employed a DNA affinity precipitation assay in order to compare the DNA binding ability of the WT Fli1 and Fli1 ΔCTA in vitro. For the experiment, Fli1 and Fli1 ΔCTA were transfected into 293T cells. Fli1 ΔCTA protein was consistently expressed at higher levels, suggesting a greater stability of the truncated Fli1 protein (Fig. 4D). Furthermore, the DNA binding ability of Fli1 ΔCTA was also increased in comparison to WT Fli1 (Fig. 4D). To determine if the increase of DNA binding of Fli1 ΔCTA affects the DNA binding status of other transcription factors in vivo, we carried out a ChIP assay for Sp1 and Ets1 in mouse fibroblasts. As shown in Fig. 4E, the DNA occupancy of Sp1 was very similar in WT and Fli1ΔCTA/ΔCTA fibroblasts, while the DNA binding of Ets1 was decreased in Fli1ΔCTA/ΔCTA fibroblasts. Since it was previously shown that Fli1 and Ets1 compete for the same Ets binding site on the COL1A2 promoter in human dermal fibroblasts (6), this observation supports the notion of the increased binding ability of Fli1 ΔCTA to the collagen promoter in vivo.

Together, these data suggest that derepression of collagen genes in Fli1ΔCTA/ΔCTA fibroblasts is not caused by the inability of truncated Fli1 to bind to the collagen promoter but most likely is a result of an aberrant transcriptional activity of Fli1 lacking its CTA domain.

Loss of repressor function of Fli1 ΔCTA is associated with altered protein-DNA and protein-protein interactions. We have recently shown that the DNA binding ability of Fli1 is regulated via p300/CBP-associated factor (PCAF)-mediated acetylation (2). The increased DNA binding of Fli1 ΔCTA raised the possibility that the absence of the CTA domain may interfere with the ability of PCAF to properly acetylate truncated Fli1. To examine this point, we cotransfected Fli1 ΔCTA together with PCAF into the 293T cells. As shown in Fig. 5A, PCAF induced robust acetylation of Fli1 ΔCTA. We have...
previously shown that Fli1 acetylation at lysine 380, which is located between the DNA binding and the CTA domains, induces release of Fli1 from the COL1A2 promoter (2). Furthermore, other studies have shown that the mechanism whereby acetylation at such a site regulates the DNA binding could involve induction of conformational change in the target protein (9, 20). To examine the possibility that the absence of the C-terminal domain affects the dissociation of acetylated Fli1 from the DNA, we used a DNA affinity precipitation assay. Consistent with our previous findings (2), overexpression of PCAF decreased the DNA binding ability of WT Fli1 by more than 95% (Fig. 5B). In contrast, the DNA binding of Fli1 ΔCTA was decreased by about 50%. Together, these results indicate that absence of the CTA domain does not interfere with acetylation of Fli1 by PCAF, but the process of Fli1 dissociation from the COL1A2 promoter is deregulated in Fli1 mutant protein.

We next investigated the effect of WT and Fli1 ΔCTA on the activity of COL1A2 promoter. In contrast to WT Fli1, which inhibited COL1A2 promoter activity in a dose-dependent manner, Fli1 ΔCTA stimulated COL1A2 activity (Fig. 5C). We hypothesized that the loss of the inhibitory activity of truncated

FIG. 4. Collagen production is increased in skin fibroblasts isolated from Fli1<sup>ΔCTA/ΔCTA</sup> mice. (A) Quantitative PCR analysis of fibrillar collagen mRNAs in fibroblasts isolated from skin of WT (white bars) and Fli1<sup>ΔCTA/ΔCTA</sup> (black bars) mice. Values are the means ± standard deviations from at least 3 mice in each group. *, statistically significant values. (B) Western blotting of type I collagen produced by fibroblasts isolated from skin of WT and Fli1<sup>ΔCTA/ΔCTA</sup> mice. (C) Formaldehyde cross-linked chromatin from Fli1<sup>+/+</sup> and Fli1<sup>−/−</sup> MEFs (left) and Fli1<sup>ΔCTA/ΔCTA</sup> skin fibroblasts (right) was subjected to ChIP experiments. Immunoprecipitations were performed using polyclonal Fli1 antibody. Immunoglobulin G (IgG) was used for negative control. Input indicates PCR performed on DNA without any immunoprecipitation. After isolation of bound DNA, PCR was performed using primers spanning the region of bp −256 to +2 of the endogenous Col1a1 promoter, bp −889 to −727 of the Col1a2 promoter, bp −780 to −604 of the Col3a1 promoter, bp −383 to −234 of the Col5a1 promoter, and bp −314 to −153 of the Col5a2 promoter. (D) Expression vectors encoding untagged human WT Fli1 and human Fli1 ΔCTA were transfected into 293T cells for 48 h. Nuclear extracts were incubated with COL1A2 biotin-labeled oligonucleotides. Proteins bound to these nucleotides were isolated with streptavidin-coupled agarose beads, and Fli1 was detected by immunoblotting with anti-calmodulin binding peptide antibody. The levels of Fli1 in cell lysates were determined by Western blotting. (E) Chromatin was isolated from cultured mouse dermal fibroblasts and immunoprecipitated using the indicated antibodies or control IgG. After isolation of bound DNA, PCR amplification was carried out using mouse α1(I) collagen (Col1a1) promoter-specific primers. Input DNA was taken from each sample before addition of an antibody. Data are representative of three independent experiments.
Fli1 may be related to the altered interaction with the transcription factors regulating COL1A2 promoter. Sp1 is a potent activator of COL1A2 promoter, and we have previously shown that Fli1 forms complexes with Sp1 (14). Therefore, we investigated if the interaction with Sp1 is affected in the Fli1/ΔCTA construct. To this end, we performed immunoprecipitation using nuclear extracts prepared from Fli1/ΔCTA fibroblasts and WT Fli1 fibroblasts. As shown in Fig. 5D the interaction of Fli1/ΔCTA with Sp1 was markedly decreased compared with WT Fli1. These results suggest that loss of the formation of transcription repressor complex contributes at least in part to the impaired repressor activity of Fli1 ΔCTA.

DISCUSSION

Collagen fibrils are the most abundant components of the extracellular matrix in the skin. In healthy skin under physiologic conditions, fibrillar collagen turnover is tightly regulated and occurs only at a low level to maintain proper tissue homeostasis. Disruption of this homeostasis leads to various pathological conditions associated with enhanced collagen deposition and scar tissue formation. At any given time, only a small proportion of fibroblasts, mainly in the subepidermal layer, express type I collagen mRNAs at detectable levels in healthy adult skin (17), suggesting that transcriptional repression plays a central role in controlling collagen biosynthesis in the skin in vivo. Specific mechanisms involved in this process have only begun to be elucidated. Our study demonstrates for the first time that Fli1 functions as a transcriptional repressor of fibrillar collagen genes in mouse skin. Expression levels of the fibrillar collagens were markedly elevated in mice with the homozygous deletion of the CTA domain of Fli1 gene (Fli1/ΔCTA/ΔCTA). Elevated expression of fibrillar collagens was maintained by fibroblasts cultured from the skin of Fli1/ΔCTA/ΔCTA mice, indicating that the absence of functional Fli1 in fibroblasts is sufficient to alleviate repression of collagen genes. The direct role of Fli1 in regulation of the type I collagen was further confirmed by ChIP analysis. These results establish Fli1 as a key regulator of the collagen gene homeostasis in the skin. Moreover, this study demonstrates that the C-terminal domain of Fli1 plays a pivotal role in collagen gene repression in vivo. Whereas previous in vitro studies have suggested that this domain is associated with gene-specific repression, our work demonstrates for the first time that it is acting as a repression domain in the context of collagen in fibroblasts both in vitro and in vivo.

Collagen biosynthesis and deposition constitute an enzymatically regulated multistep process that involves synthesis and
assembly of procollagen molecules, secretion into the extracellular space, and formation of stable collagen fibrils (23). Fli1 is a repressor of two enzymes involved in collagen fibrillogenesis, P4H (17) and Plod2, whereas expression of other enzymes including extracellular collagen processing enzymes BMP-1 and ADAMTS2 are regulated in a Fli1-independent manner (Y. Asano and M. Trojanowska, unpublished observations). Limiting the amount of these enzymes may explain the observed modest increase in collagen protein deposition despite highly elevated collagen mRNAs in the skin of Fli1 mutant mice. Alternatively, impaired fibrillogenesis may render collagen fibrils more susceptible to proteolytic degradation. Based on the existing data, we are not able to explain desynchronized expression of collagen mRNA and protein in Fli1ΔCTAΔCTA mice, and this important question would require further study.

We have observed that decorin, fibromodulin, and lumican were downregulated in response to reduced levels of Fli1, suggesting that Fli1 may be a positive regulator of these genes. Further studies are needed to determine whether these genes represent direct targets of Fli1. Interestingly, however, our recent study has shown that decorin, fibromodulin, and lumican were also downregulated in ovariectomized mice (19), suggesting that in this case Fli1 function may require estrogen receptor either through physical interaction or through cooperation on gene promoters. Alternatively, Fli1 may regulate expression of estrogen receptors in fibroblasts. Initial analysis of collagen architecture has revealed irregularities in the shape and diameter of collagen fibrils in the skin of Fli1ΔCTAΔCTA mice. Fibril abnormalities may be related to the decreased expression levels of SLRPs in mutant mice. Previous studies of the decorin, lumican, and fibromodulin knockout mice have demonstrated that each of these small proteoglycans plays a unique role in regulating collagen fibrillogenesis although in general they have inhibitory effects on collagen fibril diameter (4, 31). The physiological role of SLRPs is not limited to regulation of collagen fibrillogenesis; thus, their reduced presence may also contribute to other aspects of matrix regulation.

This study has begun to elucidate the role of the C-terminal domain in the Fli1-mediated repression of collagen genes. According to our previous studies, upon binding to the COLIA2 promoter, Fli1 acts as a potent transcriptional repressor. Fli1 DNA binding ability is regulated through PCAF-dependent acetylation at lysine 380. Acetylated Fli1, e.g., in response to transforming growth factor beta stimulation, dissociates from the collagen promoter (2). In the current study, we show that the CTA domain of Fli1 plays a key role in this repression. Whereas Fli1 lacking the CTA domain binds efficiently to the COLIA2 promoter, it no longer functions as a repressor. Further study is needed to fully understand the role of the CTA domain in transcriptional repression of collagen genes; however, this study suggests that Fli1 interaction with Sp1 may contribute to this repression. We have also observed that the ability of acetylated Fli1 to dissociate from the DNA was compromised in Fli1 ΔCTA. One possibility is that additional post-translational modifications such as phosphorylation of residue(s) located in the CTA domain are required for this process. Alternatively, the CTA domain may be necessary for the acetylation-induced conformational changes in the Fli1 molecule, which are required for efficient dissociation.

In conclusion, this study strongly supports the critical role of Fli1 in the process of collagen fibrillogenesis as a regulator of decorin, fibromodulin, and lumican expression. Fli1 function is also necessary for the maintenance of the repressed state of the fibrillar collagen genes. Given the complexity of the collagen gene regulation, other factors will likely contribute to this process, either as part of the Fli1 protein complex or through independent pathways. Future studies will be directed at the generation of better mouse models such as transgenic mice with fibroblast-specific deletion of Fli1, which will allow better characterization of the gene program regulated by Fli1 in the skin. The results of this study support the notion that the absence of Fli1 plays a pathological role in scleroderma skin fibrosis by regulating selected aspects of collagen fibrillogenesis. Lesional systemic sclerosis skin also contains irregular collagen fibrils with the abnormally thick or very thin fibrils, indicating a defect in the process of fibrillogenesis in systemic sclerosis patients (7, 32). Furthermore, elevated expression of P4H and Plod2 has been observed in scleroderma fibroblasts (16, 39). Our analysis also indicates that, in addition to transcriptional derepression of collagen genes, additional factors are needed to fully elaborate the phenotype of fibrosis. The Fli1 deletion mutant model should in the future help identify these factors by multiple mutant analyses.

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