Fli-1 inhibits collagen type I production in dermal fibroblasts via a Sp1-dependent pathway

Running title: Fli-1 inhibits COL1A2 transcription

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ABSTRACT

Fibrosis is characterized by the excessive deposition of extracellular matrix (ECM), especially collagen. Since Ets factors are implicated in physiological and pathological ECM remodeling, the aim of this study was to investigate the role of Ets factors in collagen production. We demonstrate that the expression of collagenous proteins and collagen α2(I) (COL1A2) mRNA was inhibited following stable transfection of Fli-1 in dermal fibroblasts. Subsequent analysis of the COL1A2 promoter identified a critical Ets binding site (EBS) which mediates Fli-1 inhibition. In contrast, Ets-1 stimulates COL1A2 promoter activity. In vitro binding assays demonstrate that both Fli-1 and Ets-1 form DNA-protein complexes with sequences present in the COL1A2 promoter. Furthermore, Fli-1 binding to the COL1A2 is enhanced via Sp1-dependent interaction. Studies using Fli-1 dominant interference and DNA binding mutants indicate that Fli-1 inhibition is mediated by both direct (DNA binding) and indirect (via protein-protein interaction) mechanisms and that Sp1 is an important mediator of the Fli-1 function. Furthermore, experiments using Gal4 system in the context of different cell types, as well as experiments with the COL1A2 promoter in different cell lines, demonstrate that the direction and magnitude of the effect of Fli-1 is promoter and cell context specific. We propose that Fli-1 inhibits COL1A2 promoter activity by competition with Ets-1. In addition, we postulate that another factor (co-repressor) may be required for maximal inhibition following recruitment to the Fli-1/Sp1 complex. We conclude that the ratio of Fli-1 to Ets-1 and the presence of co-regulatory proteins ultimately control ECM production in fibroblasts.
INTRODUCTION

ECM remodeling is a complex and tightly regulated process that occurs during embryogenesis, female reproductive cycle, angiogenesis, and wound repair. However, in the majority of normal adult tissues only limited turnover of the ECM takes place. In contrast, in many pathological conditions the balance between ECM synthesis and degradation is disrupted, leading to abnormal ECM remodeling. Excessive ECM breakdown is associated with rheumatoid arthritis, osteoarthritis, periodontitis, as well as tumor invasion and metastasis (1-4), whereas excessive ECM deposition occurs in fibrotic diseases such as scleroderma, liver cirrhosis, and glomerulosclerosis (5-7). The type I collagen, a product of two coordinately regulated genes, \( \alpha_1(\text{I}) \) (COL1A1) and \( \alpha_2(\text{I}) \) (COL1A2), is a predominant ECM component of the fibrotic lesion. Studies using diverse experimental systems have indicated that transcriptional regulation of the type I collagen genes is species and tissue specific (5). The COL1A2 promoter has been extensively used as an experimental model system to delineate transcriptional regulation of the collagen gene in human tissues. This promoter constitutively exhibits high levels of expression in activated dermal fibroblasts, which are “turned on” by routine in vitro tissue culture conditions such as growth on plastic in the presence of serum. Previous studies have identified the ubiquitous factors, Sp1 and Sp3, and CBP as primary activators of this promoter (8-11). In contrast, very little is currently known about the nature of the transcriptional repressors that may counteract or modulate the potent stimulatory effects of Sp1 and Sp3 on this promoter. The activity of the COL1A2 promoter can be further modulated by transcriptional factors mediating cytokine responses. Thus, Smad3 in cooperation with p300/CBP mediates activation of this promoter by TGF\( \beta \) (8,12,13), while C/EBPs as well as NF-\( \kappa \)B appear to be involved in TNF\( \alpha \) inhibition of the COL1A2 activity (14,15). Moreover, recent studies provide the evidence for Sp1 as a critical mediator of TGF\( \beta \) induction of this promoter via interaction with Smad3 (8,16).

Ets proteins comprise a family of transcription factors with over 30 members identified to date which are characterized by the presence of a highly conserved DNA binding domain, termed the ets domain (17). Ets proteins are grouped into subfamilies based on the sequence similarity of the ets domain and its location within the protein, as well as additional sequence similarities (17). Fli-1 and the closely related Erg are the members of the ERG subfamily. Extensive in vitro studies, as well as the data obtained from various Fli-1 null mice, support a crucial role for Fli-1 in hematopoiesis (18). Fli-1 is also expressed in embryonic tissues at the
sites of cell migration and epithelial-mesenchymal transition, suggesting additional functions during development (19). It may be relevant that Fli-1 is an inducer of tenascin-C (TN-C), an ECM molecule, which is also expressed at the sites of cell migration (20). However, the specific role of Fli-1 in these processes is presently not well understood. Targeted disruption of the Fli-1 gene resulted in hemorrhage into the neural tube and the ventricles of the brain at E11, resulting in embryonic death shortly thereafter. In addition to hematopoietic defects, these mutant embryos demonstrated a disruption of basement membrane tissues (18).

There is increasing evidence that some Ets proteins, particularly the members of Ets-1 and E1AF/PEA3 subfamilies, play an important role in regulating transcriptional activation of ECM degrading enzymes, including serine proteases and matrix metalloproteinases (MMPs) (21,22). Extensive previous studies of polyoma virus enhancer as well as uPA (urokinase plasminogen activator) and several MMP promoters have established a paradigm for the role of Ets in regulation of this group of genes (23). These promoters contain functional EBS(s) (ets binding site) and AP-1 site(s). The members of the Ets and Jun families that bind to the Ets-AP-1 composite element are also targets of the Ras-MAP-kinase signaling pathway (24). Thus, the transcriptional activation of this group of genes depends on the nature of interacting proteins (both Ets and AP-1 are multiprotein families) as well as the exogenous stimuli activating the Ras cascade (25).

Based on our recent observation that a matrix protein, TN-C, is also regulated by Ets factors (20), we postulated that Ets role in ECM remodeling may not be limited to regulation of the degradative pathways, but may also include regulation of synthesis of the ECM proteins (26). To test this hypothesis we selected a well-characterized system with physiological and pathological relevance, the human COL1A2 gene. In the present study we demonstrate that the collagen gene is indeed a target for Ets factors. Significantly, we provide evidence that Fli-1 acts as a transcriptional repressor of this gene, while Ets-1 acts as an activator. Furthermore, we show that cellular context plays an important role in defining both the magnitude and direction of response to Fli-1.
MATERIALS AND METHODS

Cell culture and generation of stable transfectants. Human dermal fibroblast cultures were established from newborn foreskins obtained from delivery suits of local hospitals. Foreskin tissue was dissociated enzymatically by 0.25% collagenase type I (Sigma) and 0.05% DNase (Sigma) in DMEM with 20% fetal calf serum (FBS) (Life Technologies). Human mesangial cells were purchased from Clonetics (Walkersville, MD). Hep G2 and HeLa cells were purchased from American Type Culture Collection (Manassas, VA). Fli-1 and control stable transfectants were generated in human foreskin fibroblasts transfected by the Calcium Phosphate technique with either pSG5 Fli-1 or pSG5 control vector and grown in the presence of 100-200 µg/ml of Geniticin (Gibco-BRL). Individual antibiotic resistant colonies were expanded and analyzed for the expression of Fli-1 protein.

Procollagen analysis by 3H-proline incorporation, SDS-PAGE and autoradiography. Analysis of labeled proline incorporation into secreted protein was performed as previously described (27). Fibroblasts were plated in 12-well plates and grown to visual confluency. Medium was changed to serum-free medium (DMEM) supplemented with 50 mg/ml of ascorbic acid and 64 mg/ml of β-amino propionitrile for duration of the experiment. After 24 hours incubation in serum-free medium, 10 µCi/ml of L(2,3,4,5 3H)proline (specific activity 3.6 Tbq/mmol) (Amersham) was added for 24 hours. Medium was harvested from each well and cells were trypsinized and counted. Medium was dehydrated in a SpeedVac (Savant) and resuspended in SDS/DTT sample buffer and boiled to denature. The volume of sample buffer added for resuspension was normalized according to the measured cell count of each well. After electrophoresis, gels were enhanced by immersion in 2,5-diphenyloxazole (PPO) and visualized by autoradiography.

Western blot. To determine the levels of expression of Ets-1 and Fli-1 proteins, total cell lysates (100 µg/lane) were used. To detect Ets-2 protein, nuclear extracts (35 µg/lane) were used. Samples were electrophoresed in 12% SDS-polyacrylamide gel and transblotted onto PVDF membrane (Millipore). After blocking with 5% milk, the membranes were incubated with primary antibodies, followed by incubation with HRP-conjugated secondary antibodies, and
washing with TBS buffer. Immune complexes were detected using enhanced chemiluminescence (Amersham). Monoclonal anti-Ets-1 antibody was obtained from Transduction Laboratories, polyclonal anti-Ets-2 antibody was obtained from Santa Cruz, and polyclonal anti-Fli-1 antibody was previously described (18).

**Northern blot.** Total RNA was extracted from confluent fibroblasts and analyzed by northern blotting as previously described (27). Filters were sequentially hybridized with radioactive probes for α2(I) procollagen and glyceraldehyde-3-phosphate dehydrogenase. The filters were scanned with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Plasmid constructs.** The –353 COL1A2 construct containing sequences between –353 and +58 linked to the CAT reporter gene was described previously (10). The –353 COL1A2/LUC was generated by recloning the –353 to +58 promoter fragment into pGL2 basic (Promega). The EBS promoter mutants were constructed by replacing guanosine in the EBS core sequence with thymidine using the –353 COL1A2 promoter construct as a template with the use of QuikChange Site-directed Mutagenesis Kit (Stratagene) according to manufacturer’s specifications (Table I). EBSQM used in EMSA contains EBS 1-4 mutations. The COL1A2 promoter AP1 binding site mutation was generated as previously described (28). Fli/W321R contains a single amino acid mutation which abolishes DNA binding. Fli/DBD contains DNA binding domain (267-384). pSG5Fli-1, pSG5Ets-1, pSG5Ets-2 were previously described (20). Gal4-Sp1 and Gal4-Sp3 were a generous gift of G. Suske (Marburg University, Germany). PG5luc vector was purchased from Promega.
Table I

| EBSM | WT sequence   | Mutation               |
|------|---------------|------------------------|
|      |               |                        |
| EBSM |               |                        |
| 1,2  | GTTTCAAACTTGGAAAGGG (-320) | GTTTAAAAACCTTTAAGGG (-320) |
|      | AAACTTGGAA    |                        |
|      | AGGG (-300)   |                        |
|      |               |                        |
| EBSM |               |                        |
| 3    | CGGGAGGATGC (-290) | CGGGAGTATGC (-290) |
|      | (-279)        |                        |
|      |               |                        |
| EBSM |               |                        |
| 4    | AGAGTTTCCCCCT (-250) | AGAGTTTAAACCT (-250) |
|      | (-238)        |                        |
|      |               |                        |
| EBSM |               |                        |
| 5    | AAGAATGGAACCA (-207) | AAGAATTTAACCA (-207) |
|      | (-194)        |                        |

Cell culture transfection and reporter gene assays. Transient transfections with the indicated reporter, expression, and control constructs were performed in duplicates in six well plates using FuGene 6 reagent (Roche Biochemicals) according to manufacturer's specification. Transfections were repeated at least four times using two different plasmid preparations. CAT and luciferase activities in aliquots containing equal amounts of protein were determined 48 h post-transfection.

Preparation of nuclear extracts. Nuclear extracts were prepared according to Andrews and Faller (29) with minor modifications. In vitro transcribed and translated human Fli-1, Ets-1, and Ets-2 proteins were prepared using TNT Coupled Reticulocyte Lysate System (Promega).

DNA binding assays. DNA mobility shift assays (EMSA) were performed with 32P-labeled probes as previously described (30). Briefly, 7 μg of nuclear extracts were incubated for 30 min on ice in 24 μl of the binding buffer (20 mM HEPES-KOH, pH 7.9, 50 mM KCl, 1 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethyl-sulfonyl fluoride, 10% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) containing 50 000 c.p.m.
labeled probe and 2 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia). In some assays double-stranded competitors (50-fold molar excess) or antibodies were preincubated with nuclear extracts 30 min prior to addition of radioactive nucleotide probe. Polyclonal anti-Sp1 and anti-Sp3 antibodies were purchased from Santa Cruz Biotechnology, and monoclonal anti-Ets-1 from Transduction Laboratories. Polyclonal anti-Fli-1 antibody was previously characterized (18). Separation of free radiolabeled DNA from DNA-protein complexes was carried out on a 5% nondenaturing polyacrylamide gel. Electrophoresis was performed in 0.5xTris-borate electrophoresis buffer at 250 V at 4°C. The gels were dried and exposed to X-ray film at –80°C.

**Statistical analysis** The Mann-Whitney U-test or Wilcoxon test was used to determine statistical significance.
RESULTS

_Fli-1 inhibits production of collagenous proteins and mRNA in human dermal fibroblasts_

In the first set of experiments we asked whether Fli-1 regulates ECM production by generating stable Fli-1 transfectants in human dermal fibroblasts. Several clones, which exhibited elevated levels of Fli-1 protein were selected for further investigation (Fig. 1A). Overexpression of Fli-1 in dermal fibroblasts did not affect their proliferative rate (Fig. 1B). To assess the effect of Fli-1 on the production of collagenous proteins, conditioned media from cells metabolically labeled with \(^{3}H\) - proline were analyzed by SDS-PAGE. As shown in Fig. 1C, increased expression of Fli-1 protein inversely correlated with the production of collagenous proteins. These data indicate that elevated expression of Fli-1 has a potent inhibitory effect on the newly synthesized collagenous proteins. Decreased levels of the collagenous proteins in conditioned media may result either from the activation of the degradative pathways (e.g. MMPs), or decreased collagen synthesis, or both. To determine whether Fli-1 directly affects collagen gene expression, COL1A2 mRNA levels were compared in Fli-1 stable transfectants and control clones. As shown in Fig. 1D and E, Fli-1 decreased COL1A2 mRNA levels by at least 1.5 fold suggesting that its inhibitory effects occurs, at least partially, via regulating collagen mRNA expression levels.

_Fli-1 inhibits COL1A2 promoter activity_

The effect of Fli-1 on the activity of the COL1A2 promoter was examined next. Consistent with the effect of Fli-1 on the COL1A2 mRNA levels Fli-1 inhibited COL1A2 promoter activity in a dose-dependent manner (Fig. 2A and B). To map the Fli-1 response element in the COL1A2 promoter, we utilized previously generated promoter deletion constructs (10). The strongest inhibition (2.8 fold) was observed with the –3.5 kb COL1A2 construct, whereas the –353 deletion was inhibited about 1.7 fold. The –264 deletion produced variable results, while the –186 and -108 deletions were modestly stimulated by Fli-1 (Fig. 2C). Thus, the inhibitory effect of Fli-1 was mediated by at least two separate COL1A2 promoter regions, one region located between bp –3500 and bp –772, and the second region, between bp –353 and bp –185. Since the proximal 353-bp region of the COL1A2 promoter is well characterized, we focused on this region to further delineate the molecular mechanism of the COL1A2 repression by Fli-1. Inspection of the nucleotide sequence of this promoter region revealed a presence of
five putative Fli-1 response elements (termed EBS 1-5) located at bp -317, -307 –284, -244, and -200 (Fig. 2D). Significantly, EBSs 1-3 are located within the previously identified 60-bp DNAse-1 -protected region (-322 to –262) that included three Sp1 binding sites (30).

**EBS 3 and Sp1/Sp3 binding sites function as Fli-1 response element**

To determine which of the EBSs were responsible for mediating the Fli-1 inhibition, the putative EBSs were mutated individually and in combination (as described in the methods section, see Table I). The COL1A2 –353 constructs carrying mutated GGAA motifs were tested in transient transfection assays. Substitution mutation in the EBS 3 significantly reduced Fli-1 inhibition of the COL1A2 promoter, while the mutations in other EBSs had no effect. (Fig. 3A). Since Ets factors are known to cooperate with AP-1 in regulation of uPA and several MMP promoters (31), we asked whether previously characterized AP-1 site (28) in the COL1A2 promoter could be involved in the Fli-1 inhibition. The inhibitory effect of Fli-1 on the COL1A2 promoter was not affected by mutation of the previously characterized AP-1 response element. However, the COL1A2 promoter containing mutations in the three Sp1/Sp3 binding sites surrounding EBS 3 (see Fig. 2D) become significantly less responsive to the inhibitory effects of Fli-1 (Fig. 3B). This is unexpected since Sp1/Sp3 have been shown to be potent activators of this promoter and their absence should have resulted in further decrease of the promoter activity. In fact, promoter mutations in either Sp1/Sp3 or AP-1 binding sites result in a decrease of the basal promoter activity; however only mutations in the Sp1/Sp3 binding sites affect the inhibitory response to Fli-1. This unexpected result suggests that Sp1/Sp3, in this promoter context, may be considered co-repressors for Fli-1.

**Fli-1 does not compete with Sp1 for DNA binding**

To further examine the possible functional interactions between Fli-1 and Sp1/Sp3, we first evaluated whether Fli-1 could directly interfere with Sp1/Sp3 DNA binding. To test this potential mechanism, we compared binding of the Sp1 and Sp3 to the –307 to –269 bp COL1A2 promoter fragment (39-bp oligomer) carrying all three Sp1 binding sites and either wild-type (WT) or mutated EBS 3. EMSA were performed using nuclear extracts from human fibroblasts and a radiolabeled 39-mer probe (Fig. 4A). In agreement with previously published data (9,30), several specific DNA-protein complexes were formed, which could be competed by an excess of unlabeled wild-type probe (lane 2). One of the complexes was not competed by an excess of
probe harboring EBS 3 mutation (lane 3) suggesting that the binding protein(s) in this complex are related to Ets factor(s). Furthermore, formation of the same complex was diminished when 39-mer containing mutated EBS 3 was used as a probe (lane 4). The presence of Sp1 and Sp3 in the complexes formed with the wild type and mutated probes were directly analyzed by western blotting with Sp1 and Sp3 antibodies. As shown in Fig. 4B, the EBS 3 mutation did not affect either Sp1 or Sp3 binding, suggesting that competition for DNA binding does not contribute to the inhibitory effect of Fli-1.

Fli-1 interacts directly and indirectly with the COL1A2 promoter.

To examine interaction of Fli-1 with the collagen promoter we used the –348 to –234 bp fragment of the COL1A2 promoter and nuclear extracts from human dermal fibroblasts. This promoter fragment was shown in our previous footprinting analyses to contain a large (over 60 bp) protected area, indicative of a multiprotein complex interacting with this promoter region. Sp1 and Sp3 have been identified as the components of this complex (9). Using EMSA we confirmed interaction of Sp1 and Sp3 with the –348 to –234 bp promoter region (Fig. 5A, lane 3). Fli-1 also binds to this promoter region as indicated by a supershift with the anti-Fli-1 antibody (lane 4). The ability of anti-Fli-1 antibody to shift Sp1 and Sp3 complexes in addition to Fli-1 complex, suggests the occurrence of protein-protein interaction between these proteins. To assess the effects of mutations in EBS on binding of Fli-1, we used the –348 to –234 fragment containing mutations in EBS 1-4. Although functional assays indicated that EBS 3 mediates Fli-1 response (Fig. 3) we chose to mutate other putative EBSs to test the possibility of binding to weaker potential sites. Neither Fli-1 nor Sp1 binding to this promoter region were affected by EBS 1-4 mutations (Fig. 5B, lanes 4 and 5). Quantitation of the Fli-1 supershift band from 9 independent experiments show no significant difference between Fli-1 binding to the wild-type probe and the probe containing EBS mutations (EBSQM).

To determine the role of Sp1/Sp3 in Fli-1 interactions with the COL1A2 promoter we used as a probe the promoter fragment in which all three Sp1 binding sites were mutated. As previously shown, these mutations abolish Sp1/Sp3 binding (Fig. 5B, lane 5). Significantly, Fli-1 binding was also substantially decreased with this probe as indicated by diminished supershift with anti-Fli-1 antibody (Fig. 5B, lane 6). Quantitative analysis of 6 independent experiments shows a statistically significant 25% (p<0.05) decrease in Fli-1 binding between wild type probe and probe containing mutations in Sp1/Sp3 sites. Taken together, these in vitro binding data
indicate that Fli-1 interacts with the COL1A2 promoter even in the absence of EBSs. On the other hand, the binding of Sp1/Sp3 to this promoter region appears to facilitate Fli-1 binding.

To further explore this dual mechanism, we utilized two Fli-1 mutants: a Fli/W321R mutant harboring a single amino acid mutation in the Ets domain which abolishes its ability to bind DNA (32), and D.K. Watson, unpublished observations), and a deletion mutant containing only the Ets domain (Fli/DBD). The effects of overexpression of these two Fli-1 mutants on the COL1A2 promoter were investigated (Fig. 6). In comparison to the wild type Fli-1, both mutants caused more modest but consistently reproducible decreases in the COL1A2 promoter activity, suggesting that direct (via DNA binding) and indirect (via protein-protein interaction) mechanisms contribute to the effects of Fli-1 on the COL1A2 promoter.

**Ets-1 and Fli-1 have antagonistic effects on the COL1A2 promoter and compete with each other in regulation of the COL1A2 promoter activity**

What is the molecular mechanism for Fli-1 mediated repression? Human dermal fibroblasts express several Ets factors, including Fli-1, Ets-1 and Ets-2 (Fig. 7A). In contrast to Fli-1, Ets-1 and Ets-2 stimulated the COL1A2 promoter in dermal fibroblasts. Ets-1 stimulated the COL1A2 promoter with slightly higher potency than Ets-2 (data not shown) and was selected for further investigation. While, we have previously shown that GABP α and β are expressed in dermal fibroblasts and that GABP α /β are activators of the tenascin-C promoter in fibroblasts (20), it is unlikely that GABP is involved in the COL1A2 regulation. GABP α+β, which binds to DNA as heterotetramer, requires two tandem repeats of the GGA motif, which are not present in the proximal region of the COL1A2 gene.

To assess the possibility of competition between Ets-1 and Fli-1 for the COL1A2 promoter, a constant amount of Fli-1 and the COL1A2/LUC was cotransfected with increasing concentrations of Ets-1 into dermal fibroblasts (Fig. 7B). Ets-1 was able to reverse the inhibitory effects of Fli-1 in a dose-dependent manner, suggesting that these two factors may compete for binding to the COL1A2 promoter. Consistent with this model, Ets-1 binding to the COL1A2 promoter was observed by EMSA (however, Ets-1 binding seemed to be less prominent than that observed with Fli-1) (Fig. 5C).

To compare binding affinity of Fli-1 and Ets-1 to the COL1A2 promoter, the in vitro translated Ets-1 and Fli-1 were reacted with the short promoter fragments containing individual EBSs in the presence or absence of the corresponding antibodies. The Fli-1 protein did not react
with any of the promoter fragments (data not shown), while the Ets-1 bound weakly only to the promoter fragment containing EBS3 (data not shown). These data suggest that either Ets-1 or Fli-1 alone have low binding affinity for the COL1A2 promoter. Thus, their presence in the DNA-protein complexes most likely depends on the presence of other transcription factors interacting with this promoter. As suggested previously, the candidate cofactors may be Sp1 and Sp3.

**Ets-1 and Fli-1 differ in their ability to functionally interact with Sp1/Sp3 in the Gal4 system**

To further examine the interaction between Sp1/Sp3 and Ets factors we utilized the Gal4 system. Either Sp1 or Sp3 fused to Gal4 DBD domain transactivated Gal4-responsive promoter in human fibroblasts (Fig. 8A). Interestingly, Sp3 was a more potent activator than Sp1 in the context of the Gal4 system. Cotransfection of Ets-1 with either Gal4-Sp1 or Gal4-Sp3 further enhanced Gal4-responsive promoter activity (Fig. 8A). This supports a model for functional interaction between Sp1/Sp3 and Ets-1 in dermal fibroblasts. Surprisingly, Fli-1 was also able to synergize with the Gal4-Sp1 and Gal4-Sp3 chimeric proteins under these experimental conditions. These observations, together with our previous data using the TN-C promoter (20), suggest that Fli-1 can behave either as repressor or as activator depending on the promoter context. In contrast to fibroblasts, in Hep G2 cells, Fli-1 did not have a significant effect on either Gal4-Sp1 or Gal4-Sp3 activation potential, while Ets-1 synergized with both Sp1 and Sp3 in activating Gal4 promoter (Fig. 8B). These data suggest that cell type specific cofactors may be required to facilitate Fli-1 interactions with Sp1/Sp3. Consistent with this possibility, previous studies have demonstrated that formation of ternary complex and functional interaction between Sp1 and Ets-1 required presence of the Tax protein (33). The difference between Fli-1-Sp1 interaction observed here further suggest that distinct cofactors facilitate Ets-1 and Fli-1 interaction with Sp1/Sp3. Together, these data suggest that that repressor function of Fli-1 is dependent on cell and promoter context.

**Effects of Fli-1 and Ets-1 on the COL1A2 promoter are cell type specific**

To further examine the variable responses of different cell types to Fli-1, we utilized several cell lines of different origin in the analyses of Ets-1 and Fli-1 regulation of the COL1A2 promoter (Fig. 9A). Ets-1 stimulated the COL1A2 promoter activity in all cell lines tested. In contrast, Fli-1 showed cell line-specific effects. In human lung fibroblasts Fli-1 significantly
inhibited the COL1A2 promoter. In human mesangial cells and in Hep G2 cells Fli-1 had slight, but consistent inhibitory effect, whereas in HeLa cells Fli-1 was stimulatory for the COL1A2 promoter. We have also determined the endogenous expression of Ets-1 and Fli-1 proteins in the cells used in this experiment. Ets-1 and Fli-1 were expressed in all cell types, and although there were some differences in expression levels, the ratio of Ets-1 to Fli-1 protein appeared to be similar in all cells (Fig. 9B).
DISCUSSION

The present study establishes for the first time that Ets factors play a critical role in regulating expression of the collagen type I gene in dermal fibroblasts. The following observations support this conclusion. First, exogenous expression of Fli-1 in dermal fibroblasts led to a dramatic decrease of the newly synthesized collagenous proteins and the COL1A2 mRNA. Second, transient transfection experiments have defined a functional EBS in the COL1A2 promoter. Third, binding assays have demonstrated that Fli-1 and Ets-1 form DNA-protein complexes with sequences present in the COL1A2 promoter. Furthermore, this study has also begun to unravel specific regulatory mechanisms involved in the transcriptional control of the COL1A2 gene by Ets factors. Experiments using Fli-1 dominant interference and DNA binding mutants indicate that Fli-1 inhibition is mediated by both direct (DNA binding) and indirect (protein-protein interaction) mechanisms. Based on the functional studies with the Sp1/Sp3 promoter substitution mutants as well as the EMSA results, we postulate that functional interaction of Fli-1 with Sp1 and Sp3 is essential for the inhibitory function of Fli-1. It also appears that additional tissue-specific bridging factors are involved in these interactions. The nature of these cofactors is presently unknown. Furthermore, the competition experiments suggest that Fli-1 inhibits the COL1A2 promoter by displacing Ets-1. Together, these findings imply that the ratio of Fli-1 to Ets-1 and the presence of co-regulatory proteins may contribute to control of collagen production in fibroblasts.

Our findings suggest that in the context of the COL1A2 promoter Sp1 may play a dual role of an activator and a repressor of this promoter (Fig. 10). Moreover, Fli-1, and tissue-specific co-repressor(s) may contribute to formation of this repressor complex. Recent observations indicating direct interaction between Sp1 and histone deacetylase 1(HDAC1) further support this possibility (34). A possible mechanism explaining such dual role has been proposed for a tumor suppressor protein p53 (35,36). It has been demonstrated that p53-mediated repression of specific target genes depends on association of p53 with HDAC1 and Sin 3A (35), whereas activator status of p53 can be enhanced through acetylation by p300 (36). It is intriguing that in our system Sp1 can behave as an activator and as co-repressor in the context of the same promoter. What determines the switch between these two states remains to be elucidated. However, it has been recently shown that Sp1 is involved in mediating TGFβ activation of this promoter (8), suggesting a possible role of TGFβ induced signaling in this process.
Studies using dermal fibroblasts indicate that Fli-1 can act as a repressor in the context of the collagen promoter, while it is a potent activator for the TN-C promoter (20). What determines this dual function of Fli-1 is currently not known. Only a subset of ETS factors have repressor activity (e.g. ERF, YAN, TEL, NET) and most are generally felt to be activators of transcription. However, previous studies have demonstrated that other proteins via interaction with Ets can block their ability to activate transcription. For example, interaction of EAP1/Daxx with Ets-1 has been shown to downregulate Ets-1 mediated activation of MMP1 and BCL2 genes (37). In another system, MafB, an AP-1 like protein, has been shown to interact with the ets domain of ETS-1 and repress Ets-1 transactivation of Ets-responsive promoters (38). More importantly, Fli-1 has been shown to be an interacting partner of TEL, a member of Ets family with repressor function. Furthermore, co-transfection of Tel with Fli-1 has been shown to inhibit Fli-1 mediated transcriptional activation (39). TEL contains two autonomous repression domains suggesting two distinct repression mechanisms (40). One of the potential mechanisms involves recruitment of a repression complex including SMRT and mSin3A (41). Whether TEL is involved in Fli-1 mediated repression of the COL1A2 remains to be elucidated. Significantly however, stable overexpression of TEL in NIH 3T3 fibroblasts led to downregulation of collagen type I (42).

In support of the conclusions from our studies, it was observed that exogenous expression of EWS/FLI1 and EWS/ETV1 inhibited deposition of collagen in NIH3T3 cells (43). Significantly, we have found that a subset of lesional scleroderma fibroblast cell lines expresses reduced levels of Fli-1 protein as compared to healthy control fibroblasts, while the expression of Ets-1 remains constant. The reduced levels of Fli-1 correlate with increased collagen production by these cells (J. Czuwara-Ladykowska and M. Trojanowska, unpublished data). Thus, Fli-1 and other Ets factors may play a role in pathology of fibrotic diseases. It will be important to examine in vivo expression of Ets factors in fibrotic lesions and in animal models of fibrosis.
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FIGURE LEGENDS

Fig. 1: Characterization of stable transfectants overexpressing Fli-1. (A) Fli-1 protein levels in independent dermal fibroblast clones carrying empty vector (Neo) or Fli-1 cDNA. In the first line (c) an in vitro translated (TNT) Fli-1 was used. The expression level of Fli-1 was examined by Western blot with a polyclonal anti-human Fli-1 Ab. (B) Growth curve of Fli-1 (clone No 5) and control transfectants. (C) Fli-1 overexpression decreases collagen protein level. Newly synthesized collagenous proteins in control (Neo) and Fli-1 stable transfectants were measured in a [3H]-proline incorporation assay. Conditioned media normalized for cell number were analyzed for collagenous protein content via SDS-PAGE and fluorography. (D) Fli-1 overexpression inhibits COL1A2 mRNA expression. RNA isolated from the (empty vector) control and Fli-1 stable transfectants was analyzed simultaneously by northern blot. Blots were hybridized with probes for COL1A2 and GAPDH as the loading control. (E) A summary of quantitative analysis of the northern blots from three different clones. All values were corrected for the loading differences by normalizing to the GAPDH mRNA intensity.

Fig. 2: The effect of Fli-1 overexpression on the COL1A2 promoter activity and identification of the COL1A2 promoter region mediating Fli-1 inhibition. (A) Expression of exogenous Fli-1 protein. Dermal fibroblasts were transfected with indicated amounts of Fli-1/Flag expression vector or empty vector (pSG5) (line 1). Protein levels of Fli-1/Flag in transfected cells was analysed by immunoprecipitation with anti-Flag M2 monoclonal antibody (Sigma) and western blot of the precipitates with anti Fli-1 polyclonal antibody. Equivalent amounts of protein were used for the immunoprecipitations. (B) Dose-dependent inhibition of the –353COL1A2/CAT promoter by Fli-1. The COL1A2 promoter construct (0.1 µg) was cotransfected with increasing concentrations of Fli-1 expression vector. To ensure equal amount of cotransfected expression vectors under each condition appropriate amounts of pSG5 were added to individual cotransfections. (C) Transient transfections of human dermal fibroblasts with the indicated deletion constructs of the COL1A2/CAT promoter and either empty vector (pSG5) or Fli-1 expression vector. The bar graph represents fold induction of the promoter activity of each construct cotransfected with Fli-1 vector relative to the activity of the promoter cotransfected with empty vector (pSG5), which was arbitrarily set at 1. The average of at least four
independent experiments is shown. Fli-1 responsive element was mapped between −353 and −186. (*p<0.05). (D) Nucleotide sequence of the COL1A2 promoter region between bp−353 and bp−186. The five potential Ets binding sites (EBS) are boxed. Sp1/Sp3 binding sites are underlined.

Fig. 3: (A) Identification of a functional Fli-1 response element in the human COL1A2 promoter. The −353 COL1A2/CAT constructs harboring mutated EBSs (see Fig. 2B and Table I, methods section) were cotransfected with either Fli-1 expression vector or empty vector (pSG5). The bar graph represents fold induction of the promoter activity of each construct cotransfected with Fli-1 relative to the activity of the promoter cotransfected with pSG5, arbitrarily set at 1. Means + SE of 5 independent experiments are shown. Comparisons of COL1A2 promoter activity were made between cells transfected with empty vector and with Fli-1 expression vector (*<0.01), and between cells transfected with wild-type COL1A2 and EBS mutated COL1A2 constructs (¶<0.01). (B) Sp1/Sp3 binding is required for the inhibitory effect of Fli-1. The COL1A2/CAT promoter constructs harboring either Sp1/Sp3 triple mutations (Sp1TM) (45) or AP1 mutation (11) were cotransfected with Fli-1 expression vector or empty vector pSG5. Comparisons of COL1A2 promoter activity were made between cells transfected with wild type promoter and mutated promoter constructs (*=p<0.01). The Fli-1 inhibitory effect on COL1A2 was diminished in Sp1TM.

Fig. 4: Sp1/Sp3 binding to the COL1A2 promoter is independent of EBS 3. (A) EMSA with competitor oligonucleotides. The wild-type probe (lanes 1 to 3) or the EBS 3 mutated probe (lane 4) were incubated with human fibroblast nuclear extracts (5 µg/lane) in the presence of wild-type unlabeled competitor (50-fold molar excess) (lane 2) or EBS 3 mutated unlabeled competitor (lane 3). (B) Detection of Sp1 and Sp3 in the DNA-protein complexes. EMSA were performed with equal amounts of unlabeled wild type and EBS 3 mutated probes, followed by western blot detection with anti-Sp1 and anti-Sp3 antibodies.

Fig. 5: Fli-1 interacts with the human COL1A2 promoter. (A) EMSA with antibodies against Sp1, Sp3, Fli-1 was performed. The wild type fragment of the COL1A2 promoter (-348 to −234) (lanes 1-4) was incubated with nuclear extracts (7 µg/lane) from human dermal fibroblasts in the presence of 1 µg of specific Ab as indicated on the top. In line 2 anti-human polyvalent IgG was
used as a control. Line 1 contains probe alone. Specific DNA-protein complexes and supershifted complexes are indicated by arrows. (B) Sp1/Sp3 binding facilitates Fli-1 binding to the human COL1A2 promoter. EMSA was performed with the wild type probe (WT) (lanes 1-2), probe containing mutated EBS 1-4 (EBSQM) (lanes 3-4), and probe containing mutated Sp1/Sp3 binding sites (Sp1TM) (lanes 5-6). The probes were incubated with 7 µg of nuclear extract from dermal fibroblasts and 1 µg of specific antibodies. Fli-1 binding was significantly decreased with Sp1TM probe as indicated by diminished supershift with anti-Fli-1 antibody. (C) Ets-1 interacts with the human COL1A2 promoter. The wild type probe of COL1A2 promoter (-348 to –234) (lanes 1-4) was incubated with nuclear extracts (7 µg/lane) from human fibroblasts in the presence of 1 µg of specific Ab as indicated on the top. The arrow indicates Ets-1 supershift.

Fig. 6: Fli-1 mutants inhibit the COL1A2 promoter activity. Transient transfections were performed with Fli-1 DNA binding mutant (Fli/W321R) and Fli-1 dominant interference mutant (Fli/DBD) and the –353 COL1A2 promoter. The Fli/W321R mutant contains a single amino acid mutation, which abolishes DNA binding and the Fli/DBD mutant contains the Ets domain. Both Fli-1 mutants significantly decreased the COL1A2 promoter activity (*=p<0.05), but were less efficient than wild-type Fli-1.

Fig. 7: Ets family members expressed in human fibroblasts have distinct effects on the transcriptional activation of the COL1A2 promoter. (A) Dermal fibroblasts express Fli-1, Ets-1, and Ets-2. Lane 1, corresponding control Ets TNT product; lane 2, endogenous Ets protein. (B) Overexpression of Ets-1 stimulates the COL1A2 promoter activity and abolishes Fli-1 inhibition of COL1A2 promoter in dermal fibroblasts. Human dermal fibroblasts were transiently cotransfected with the –353 COL1A2/LUC promoter construct (0.9 µg) and either with Fli-1 (0.1 µg) or Ets-1 (0.1 µg) added individually or with constant amount of Fli-1 expression vector (0.1 µg) added together with increasing amounts of Ets-1 (0.025, 0.05, 0.1 µg) expression vector. To ensure equal amount of cotransfected expression vectors under each condition appropriate amounts of pSG5 were added to individual cotransfection. The bar graph represents fold induction of the –353 COL1A2 promoter activity cotransfected with Fli-1 or Ets-1 individually or together relative to the activity of the promoter cotransfected with pSG5, which was arbitrarily set at 1. The average ± SE from four independent experiments is shown.
Fig. 8: (A) Fli-1 and Ets-1 cooperate with Sp1 and Sp3 in activation of Gal4 promoter in dermal fibroblasts. Human dermal fibroblasts were cotransfected with 0.4 µg of a Gal4-dependent luciferase (Gal4/LUC) reporter construct and with 0.4 µg of expression constructs of fusion proteins containing the DNA binding domain of Gal4 (Gal4DBD) and Sp1 and Sp3, Gal4-Sp1 and Gal4-Sp3, respectively. In some experiments 0.1 µg of Fli-1 or Ets-1 expression vectors were also cotransfected. The bar graphs represent means ± SE of the fold induction of Gal4 promoter activity from experimental conditions relative to the activity of the Gal4 promoter cotransfected with Gal4DBD only, which was arbitrarily set at 1. (B) Ets-1 but not Fli-1 cooperates with Sp1 and Sp3 in activation of Gal4 promoter in Hep G2 cells. The same experimental conditions were used in transfections with Hep G2 cells.

Fig. 9: Transactivation of the COL1A2 promoter by Ets factors is cell context dependent. (A) Human lung fibroblasts, mesangial cells, Hep G2 and HeLa cells were transiently co-transfected with the –353 COL1A2/LUC promoter construct and Fli-1 or Ets-1 expression vectors. The bar graph represents fold induction of the COL1A2 promoter activity relative to the activity of the promoter cotransfected with pSG5, which was arbitrarily set at 1. The average ± SE of at least three independent experiments is shown (*=p<0.05). (B) Expression of endogenous Fli-1 and Ets-1 proteins in different cell types.

Fig. 10: A hypothetical model for Sp1 mediated transcriptional repression or activation. Details described in the discussion.
Figure 1, Czuwara et al
Figure 2, Czuwara et al

A

| Transfection: | SG5  | Fli-1 |
|--------------|------|-------|
| 50 ng        |      |       |
| 100 ng       |      |       |
| 200 ng       |      |       |

Fli-1/Flag

IP: anti-Flag Ab
WB: anti-Fli-1 Ab

B

![Bar graph showing fold induction of Fli-1/SG5](image)

Empty Vector +

Fli-1 -

C

![Bar graph showing fold induction for different regions](image)

Fold induction (Fli-1/SG5)

-3500
-772
-353
-264
-186
-108

Empty Vector

Fold induction (Fli-1/SG5)
Figure 2, Czuwara et al

D

-353

GGG GCTGCAGAGC ACTCCGACGT GTCCCATAGT GTTTCCAAAC

EBS1

EBS2 Sp1/Sp3 Sp1/Sp3 EBS3 Sp1/Sp3

TTGGAAAGGG CGGGGGAGGG CGGAGGGCGG

EBS4

AGGTATGCAG ACAACGAGTC AGAGTTTCCC CTTGAAAGCC

EBS5

TCAAAAGTGT CCACGTCCTC AAAAAGAATG GAAACCAATTT

AAGAAGCCAG

-180
Figure 3, Czuwara et al

A

Fold induction (FlI-1/SG5)

Empty vector  WT  EBSM 1.2  EBSM 3  EBSM 4  EBSM 5

B

Fold induction (FlI-1/SG5)

Empty Vector  WT  SplTM  APIM
Figure 4, Czuwara et al
Figure 5, Czuwara et al

A

| Ab | IgG | Sp3 | Fli-1 |
|----|-----|-----|-------|

Fli-1 supershift

Sp1/Sp3

Sp3

Fli-1

probe -348 to -234
Figure 5, Czuwara et al

B

Ab

IgG, Fli-1

Fli-1 supershift

1 2 3 4 5 6

WT EBSQM Sp1TM
Figure 6, Czuwara et al

|                | Empty Vector | Fli-1 | Fli/W321R | Fli/DBD |
|----------------|--------------|-------|-----------|---------|
| Fold Induction | 1.00         | 0.65  | 0.80      | 1.00    |

DNA binding activity:
- Fli-1: +
- Fli/W321R: -
- Fli/DBD: +
Figure 7, Czuwara et al

A

B

relative LUC activity (fold induction)

Fli1 (0.1 μg) Ets-1

EV + + + + + +

- - 0.025 0.05 0.1 +
Figure 9, Czuwara et al.

A

Lung fibroblasts

Mesangial cells

Hep G2 cells

HeLa cells

B

Fli-1

Ets-1
Figure 10, Czuwara et al

**COL1A2**

**Sp1**

**FII-1**

**HDAC 1**

**Sin3A**

**TEL**

**Repression**

**Activation**

**CBP/p300**

**Sp1**

**Ets-1**

**X**

**Y**
Fli-1 inhibits collagen type I production in dermal fibroblasts via a Sp1-dependent pathway Running title: Fli-1 inhibits COL1A2 transcription
Joanna Czuwara-Ladykowska, Fumiaki Shirasaki, Pascale Jackers, Dennis K. Watson and Maria Trojanowska

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