Ets1 Is an Effector of the Transforming Growth Factor β (TGF-β) Signaling Pathway and an Antagonist of the Profibrotic Effects of TGF-β*

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Extracellular matrix (ECM) production and turnover are tightly controlled under normal physiological conditions. Ets factors regulate matrix turnover byactivating transcription of several metalloproteinases (MMPs) and are frequently overexpressed in aggressive tumors and arthritis. Because of the prominent role of transforming growth factor β (TGF-β) in ECM synthesis, this study was undertaken to determine the possible inter-actions between Ets1 and the TGF-β pathway. Experiments using adenoviral delivery of Ets1 in human fibroblasts have established that Ets1 strongly suppresses TGF-β induction of collagen type I and other matrix-related genes and reverses TGF-β-dependent inhibition of MMP-1. Subsequent experiments utilizing COL1A2 promoter demonstrated that Ets1 in the presence of TGF-β signaling interferes with the stimulatory role of p300. To gain further insight into the mechanism of Ets1 inhibition of the TGF-β signaling, the protein levels and post-translational modifications of Ets1 after TGF-β treatment were analyzed. The level of total Ets1 protein was not affected after 24 h of TGF-β stimulation. Moreover, TGF-β did not affect either serine or threonine phosphorylation levels of Ets1. However, TGF-β induced rapid and prolonged lysine acetylation of Ets1. In addition, analyses of endogenous p300-Ets1 complexes revealed that acetylated Ets1 is preferentially associated with the p300/CBP complexes. TGF-β treatment leads to dissociation of Ets1 from the CBP/p300 complexes. Together, these findings suggest that elevated expression of Ets1 in fibroblasts fundamentally alters their responses to TGF-β in favor of matrix degradation and away from matrix deposition as exemplified by arthritis and cancer.

The extracellular matrix (ECM) is a network of macromolecules surrounding all cells and comprising collagens, proteoglycans, and multiadhesive matrix proteins. Controlled ECM synthesis, deposition, and degradation occur during embryogenesis, the female reproductive cycle, angiogenesis, and tissue repair, but in the majority of normal adult tissues, only limited turnover of the ECM takes place. In contrast, the balance between ECM synthesis and degradation is disrupted under many pathological conditions, leading to abnormal ECM remodeling. Excessive matrix synthesis and deposition is characteristic for fibrotic diseases such as systemic sclerosis, liver cirrhosis, and glomerulosclerosis. Unbalanced matrix degradation takes place in rheumatoid arthritis, osteoarthritis, as well as tumorigenesis and is associated with cartilage and bone destruction, tumor invasion, and metastasis (1).

Transforming growth factor β (TGF-β) plays an important role in tissue remodeling. TGF-β is one of the most potent inducers of ECM proteins, and its role in the pathogenesis of fibrosis is well established (2). TGF-β up-regulates ECM deposition via several different mechanisms, including stimulation of collagen synthesis, induction of the profibrotic cytokine CTGF, and up-regulation of certain ECM receptors and protease inhibitors (e.g. PAI-1, TIMP-1) (3, 4). Although the role of TGF-β in arthritis and cancer is less clear, it is believed that TGF-β plays a dual role in these pathological conditions. In rheumatoid arthritis, TGF-β is a major contributor to synovial fibroblast hyperplasia, and it is abundant in rheumatoid joints (5). Locally, TGF-β may promote reparative processes and prevent cartilage destruction. However, in chronic lesions, over-production of TGF-β contributes to ongoing damage by recruiting monocytes and promotion of angiogenesis (6).

In early stages of tumor development, TGF-β is anti-tumorigenic, acting as a growth inhibitor to epithelial cells. In many advanced tumors, TGF-β promotes tumorigenicity by stimulating angiogenesis, inducing extracellular matrix degradation, and inhibiting anti-tumor immune responses (7). The positive role of TGF-β in tumor promotion is well documented in vivo in human breast cancer, where TGF-β expression positively correlates with the rate of disease progression (8, 9). These apparently divergent abilities of TGF-β to promote tumor invasion and metastasis and its strong profibrotic activities are likely to be related to distinct properties of the target cells. For example, the altered or lost sensitivity to TGF-β of various neoplastic cells and stromal mesenchymal cells can be linked to the loss of TGF-β receptors or dysregulation of TGF-β signal transduction pathways (10, 11).

A number of in vitro and in vivo studies demonstrate that the Ets family of transcription factors plays an important role in ECM turnover. There is increasing evidence that some Ets factors, particularly members of the Ets1 and E1A/PEA3 sub-

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1 The abbreviations used are: ECM, extracellular matrix; IP, immunoprecipitation; WB, Western blot; TGF-β, transforming growth factor β; CTGF, connective tissue growth factor; MMP, matrix metalloproteinase; CBP, CREB-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; HAT, histone acetyltransferase; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase.
families, play important roles in transcriptional activation of ECM-degrading enzymes, including serine proteases (uPA) and matrix metalloproteinases (e.g., MMP-1, MMP-3, MMP-9) (12, 13). Constitutive expression of Ets proteins (v-Ets1 and c-Ets2) has been associated with cellular transformation of erythroblasts and fibroblasts (14–16), and abnormal Ets expression has been correlated with pathological processes such as tumor invasion (17, 18) and rheumatoid arthritis (19). Co-expression of Ets1, MMP-1, and MMP-3 in stromal fibroblasts has been demonstrated by immunohistochemical studies of lung carcinoma and angiosarcoma of the skin (17, 20, 21). Synovial fibroblasts are direct effectors of tissue injury and matrix remodeling in rheumatoid arthritis, based on their ability to aggressively degrade the extracellular matrix (22). In vivo studies have shown that Ets1 protein expression is elevated in synovial fibroblasts in rheumatoid arthritis tissue (19). In either dermal or synovial fibroblasts, the most potent inducers of Ets1 are proinflammatory cytokines such as TNF-α and interleukin 1, previously implicated in cartilage destruction in arthritis and present in the tumor microenvironment (6, 19, 23).

Based on our previous observations that Ets factors regulate the expression of matrix proteins such as collagen and tenascin C (24, 25), it became important to examine the consequences of Ets1 overexpression on ECM turnover in the presence of TGF-β. For these studies, we utilized human fibroblasts, because in vivo studies have demonstrated that Ets1 is expressed at elevated level in fibroblastic cells. We have identified a possible interaction between Ets1 and the TGF-β signaling pathway. Based on our results, we postulate that Ets1 is a potent and selective suppressor of TGF-β-induced genes, and therefore, that altered or lost sensitivity of stromal mesenchymal cells to TGF-β may be mediated at least in part by Ets1 overexpression.

MATERIALS AND METHODS

Cell Culture—Human dermal fibroblast cultures were established from newborn foreskins obtained from delivery suites of local hospitals. Foreskin tissue was dissociated enzymatically by 0.25% collagenase (Sigma) and 0.05% DNase I (Sigma) in Dulbecco modified Eagle’s medium (DMEM) with 20% fetal calf serum (HyClone). Cells were grown at 37 °C in a 5% CO₂ atmosphere in DMEM supplemented with 10% fetal calf serum and 50 μg/ml gentamicin (Sigma). All studies utilized cells from passages 3–8. Before stimulation with TGF-β1 and infection with adenoviruses, fibroblasts were incubated in serum-free medium for 24 h. HepG2 cells were purchased from American Type Culture Collection and cultured in DMEM with 10% fetal calf serum and gentamicin. 293A cells (QBI-293A cells) were purchased from Transduction Laboratories and seeded on 6-well plates (10⁵ cells/well) and transfected 24 h later. Transfections were repeated at least four times using two different plasmid preparations. Luciferase activities in cell lysates containing equal amounts of protein were determined 48 h after TGF-β1 stimulation using the Luciferase Assay System from Promega.

Western Blot (WB) and Immunoprecipitation (IP)—Confuent, serum-starved dermal fibroblasts cultured on 100 mm² dishes were treated with 2 ng/ml TGF-β1 for different time periods. To determine the levels of Ets1 and Smad3 and their post-translation modifications after TGF-β1 treatment, cell lysates were used for Western blotting and IP. Western blot analyses, 100 μg of total cell lysate was used. For IP, 40 μg of total cell protein were used. Cells were lysed in buffer A containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM sodium fluoride, 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, and 1 μM phenylmethylsulfonylfluoride with protease inhibitors (Mixture Set III, Calbiochem). Protein concentration was determined by BCA Protein Assay (Pierce). Complex formation was performed overnight at 4 °C using 7 μg of each antibody followed by precipitation with protein G-Sepharose (Amersham Biosciences) for 2 h in 4 °C. For IP, the following antibodies were used: monoclonal Ets1 (E44; described previously (32)), polyclonal Ets1 (C-20), polyclonal Smad2/3 (N-19 and FL-425, Santa Cruz), or monoclonal anti-FLAG antibody (M5, Sigma). Negative controls were performed using normal rabbit or mouse IgG. The immunoprecipitates were washed four times in buffer A, eluted by boiling for 5 min in 2× SDS sample buffer, and analyzed by Western blot. Samples were electroblotted in 12% SDS-polyacrylamide gel and transblotted onto polyvinylidene difluoride membranes (Millipore). After blocking with 3% milk or 3% bovine serum albumin, the membranes were incubated with primary antibodies (polyclonal Ets1 antibody, polyclonal IgG termolysin antibody (Ac-K-103, Cell Signaling Technology), rabbit anti-phosphoserine, rabbit anti-phosphothreonine (Zymed Laboratories), or mouse anti-phosphothreonine (Santa Cruz Biotechnology)) overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies, and washed with Tween/Tris-buffered saline solution. Proteins were detected using enhanced chemiluminescence (Amersham Biosciences).
Total protein was detected by Western blotting on the same membranes after stripping, using appropriate antibodies: monoclonal Ets1 and monoclonal Smad2/3 (Transduction Laboratories), polyclonal Ets1 (C-20), polyclonal Smad3 (FL-425), or monoclonal β-actin (clone AC-150, Sigma).

Co-immunoprecipitation-Recapture—Co-immunoprecipitation-recapture was performed as described elsewhere (67). Fibroblasts were lysed in nondenaturing buffer B (50 mM Tris-HCl (pH 7.4), 150 NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 0.5 mM dithiothreitol, and 1 mM pepsin) and boiled for 5 min to release bound Ets1 and Smad3. Supernatants were transferred to new tubes containing 10 volumes of buffer B without immunocomplexes were disrupted by denaturing in 1% SDS and boiling for 10 min; 150 ng of total cell lysate was used for each condition. Co-IP was performed at 4 °C overnight with 20 μg of mouse anti-human p300 antibody (PharMingen). “Free” Ets1 and Smad3 were immunoprecipitated sequentially from the supernatant using 10 μg of polyclonal Ets1 antibody (C-20) and Smad3 (FL-425, Santa Cruz), respectively. p300 immunocomplexes were disrupted by denaturing in 1% SDS and boiling for 10 min. The amount of p300 complex and free fraction was performed with p300 (N-15, Santa Cruz) and polyclonal β-actin (clone AC-150, Sigma) antibodies, respectively, in corresponding fractions. In vitro-transcribed and translated human Ets1 protein using the TNT-coupled Reticulocyte lysates system (Promega) was used as the control for WB.

Statistical Analysis—Paired analyses were performed using the Wilcoxon test to evaluate statistical significance. Data values are expressed as mean ± S.E. Statistical significance was defined as a p value of 0.05 or less.

RESULTS

Ets1 Abrogates TGF-β-dependent Stimulation of Collagenous Proteins—In the first set of experiments, we asked whether Ets1 regulates ECM production in human dermal fibroblasts in the presence of TGF-β by utilizing an adenosine expressing Ets1. To assess the effect of Ets1 on newly synthesized collagenous proteins, including procollagens type I and III and fibronec tin, conditioned media from cells metabolically labeled with [3H]proline were analyzed by SDS-PAGE. As shown in Fig. 1, A and B, collagenous proteins were induced by TGF-β in control fibroblasts infected with the GFP-adenovirus but not in cells infected with the Ets1-adenovirus. Ets1 expression almost completely abrogates the stimulatory effect of TGF-β on collagenous protein synthesis. Thus, these data indicate that Ets1 is a potent inhibitor of TGF-β-induced synthesis of collagenous proteins. Decreased levels of the collagenous proteins in conditioned media may result from the activation of the degradative pathways (e.g. MMPs), decreased collagen synthesis, or both. Overexpression of Ets1 in dermal fibroblasts did not affect cell viability or proliferation rate up to 48 h-post infection (data not shown).

Ets1 Inhibits Induction of TGF-β Target Genes—To determine whether Ets1 directly affects type I collagen gene induction by TGF-β, COL1A1, and COL1A2 mRNA levels were compared in dermal fibroblasts infected with either Ets1 or control adenovirus. As shown in Fig. 2, A–C, Ets1 suppressed TGF-β-dependent stimulation of COL1A1 and COL1A2 mRNAs. Both messages were decreased to or below basal level. These results indicate that the inhibitory effect of Ets1 on TGF-β-dependent type I collagen synthesis occurs primarily via the regulation of COL1A1 and COL1A2 mRNA levels. In addition, other TGF-β target genes, including CTGF and TIMP-1, were examined. In agreement with previously published results, TGF-β strongly induced the expression of CTGF (4) and TIMP-1 (33) in control GFP-adenovirus-infected fibroblasts. Significantly, this response was abrogated in cells overexpressing Ets1. These findings indicate that elevated Ets1 expression in human fibroblasts can inhibit the profibrotic effects of TGF-β. It is noteworthy that overexpression of Ets1 did not significantly affect basal levels of type I collagen and other ECM-related genes in unstimulated cells, suggesting that the inhibitory function of Ets1 manifests itself in the context of the TGF-β signaling pathway. Following TGF-β stimulation, Ets1 becomes a strong inhibitor of ECM synthesis, since COL1A2 and TIMP-1 mRNA levels were significantly below the control levels of expression.

Ets1 Overcomes TGF-β-dependent Inhibition of MMP-1—It has been demonstrated previously that TGF-β abrogates basal and interleukin 1β-dependent stimulation of MMP-1 in dermal fibroblasts (34, 35). On the other hand, Ets1 is a well-characterized activator of MMP-1 transcription (36). We examined the effect of Ets1 on TGF-β inhibition of MMP-1. In agreement with previous studies, we observed that TGF-β inhibits the expression of MMP-1 mRNA in fibroblasts in a time-dependent manner (Fig. 2F, insert) (34), whereas in cells overexpressing Ets1, MMP-1 mRNA levels were strongly induced (up to 30-fold) (Fig. 2, A and F). TGF-β had little effect on Ets1-dependent induction of MMP-1. Our results confirm that Ets1 is a potent activator of MMP-1 gene expression, and moreover, that
Ets1 expression is able to overcome TGF-β/H9252-dependent inhibition of MMP-1 expression. Collectively, our data indicate that Ets1 strongly suppresses TGF-β/H9252 induction of collagen type I and other matrix-related genes and reverses TGF-β/H9252-dependent inhibition of MMP-1.

**Ets1 Inhibits TGF-β-dependent Transcription**—To further analyze the interactions between Ets1 and the TGF-β signaling pathway, we utilized the COL1A2 promoter. In agreement with published observations, TGF-β stimulated the COL1A2 promoter, and this stimulation was further enhanced by overexpression of p300 (Fig. 3A) (37). In unstimulated cells, Ets1 was a weak inducer of COL1A2 promoter as shown previously by our laboratory (25). However, in TGF-β-stimulated cells, Ets1 inhibited TGF-β/p300-induced responses. Note that in the absence of TGF-β, Ets1 did not interfere with the p300-dependent up-regulation of COL1A2, suggesting that TGF-β signaling is required for inhibitory activity of Ets1. When p300 was substituted by a histone acetyltransferase-deficient mutant of p300 (p300ΔHAT), both basal transcription and TGF-β-dependent activation of COL1A2 were substantially reduced (Fig. 3B), suggesting that HAT activity of p300 is required for maximal TGF-β stimulation of this promoter.

Previous studies have shown that Ets1 positively regulates the MMP-1 promoter (12). In addition, CBP/p300 is known to bind to Ets1 and facilitate the Ets1 trans-activating function (38). We have tested the effects of TGF-β on the Ets1/p300-dependent activation of the MMP-1 promoter in HepG2 cells and dermal fibroblasts. Both cell types responded in a similar manner, but the magnitude of response was significantly greater in HepG2 cells. In contrast to the COL1A2 promoter, TGF-β did not affect the magnitude of either Ets1 or Ets1/p300 activation of the MMP-1 promoter (Fig. 3C). Interestingly, p300 lacking HAT activity was only slightly less potent that the wild type p300 in activating MMP-1 promoter either alone or in combination with Ets1 (Fig. 3D). These observations suggest that the HAT activity of p300 is not critical for the cooperation between Ets1 and p300 on the MMP-1 promoter. Together, these data suggest that Ets1 in the presence of TGF-β signaling may
Ets1 Is Rapidly Acetylated by TGF-β—Wild type p300 was more synergistic with TGF-β-mediated stimulation of the COL1A2 promoter than p300ΔHAT. Furthermore, in the presence of TGF-β signaling, Ets1 differentially affected p300-dependent stimulation of the COL1A2 and MMP-1 promoters. In light of the ability of p300/CBP to catalyze acetylation of transcription factors, we reasoned that Ets1 may be a potential target of the cellular acetylases, and thus we examined whether Ets1 is acetylated in response to TGF-β. As shown in Fig. 4A, endogenous Ets1 exhibited measurable levels of lysine acetylation in unstimulated fibroblasts, whereas acetylated Ets1 was barely detectable in HepG2 cells (Fig. 4B). Within 1 h of TGF-β treatment, prominent lysine acetylation of Ets1 was observed. In both cell types, TGF-β-dependent Ets1 acetylation was rapid and prolonged. Similarly, we have found that exogenously expressed Ets1/FLAG can be acetylated by TGF-β with kinetics similar to that of the endogenous protein (Fig. 4C). These results indicate that Ets1 is acetylated in response to the TGF-β signaling pathway.

We also examined the acetylation status of Smad2 and Smad3, which are known downstream targets of TGF-β signaling pathway and are involved in TGF-β-dependent regulation of COL1A2 in dermal fibroblasts and mesangial cells (39–42). 

As shown in Fig. 4D, Smad2 and Smad3 were not acetylated in human fibroblasts either before or after TGF-β stimulation.

Ets1 Phosphorylation Is Not Affected following Activation of the TGF-β Signaling Pathway—Ets1 is a nuclear phosphoprotein in which DNA binding and transcriptional activation are regulated by phosphorylation (43, 44). It is a well known nuclear target of Ras-MAPK and calcium signaling pathways. The Ras-Mek-Erk (extracellular signal-regulated kinase) signaling cascade targets the conserved threonine residue (Thr-38) and stimulates Ets1 transcriptional activity but does not affect DNA binding (38, 44–46). In contrast, calcium-dependent serine phosphorylation inhibits Ets1 DNA binding by reinforcing autoinhibition (44). In light of these well documented posttranslational modifications of Ets1, we examined whether Ets1 phosphorylation on threonine or serine is modulated by the TGF-β pathway. As presented in Fig. 5A, threonine phosphorylation of Ets1 was not significantly altered following TGF-β stimulation. This result is in agreement with the previously published observation that in dermal fibroblasts TGF-β does not activate MAPK (47). In addition, serine phosphorylation of Ets1 was not affected by TGF-β. The absence of serine phosphorylation is consistent with our electrophoretic mobility assays indicating that DNA binding activity is not altered following TGF-β treatment (data not shown). In contrast to Ets1, Smad2 and Smad3 were rapidly and transiently phosphoryl-
ated on serine after TGF-β treatment in dermal fibroblasts (Fig. 5B).

**TGF-β Modulates Association of Ets1 and Smad3 with the p300/CBP Complexes**—Ets1 has been shown to form a stable complex with p300/CBP independently of DNA binding (38, 48). Ets1 binds to two different domains of p300/CBP. One is in the N-terminal region between amino acids 313 and 452, and the second one is located in the proximity of the HAT domain between amino acids 1449 and 1892 (38). We next wanted to examine whether endogenous p300/Ets1 complexes were affected by TGF-β signaling. The experimental design is presented in Fig. 6A. Cells were stimulated with TGF-β for 0, 3, and 24 h, and p300 were immunoprecipitated under non-denaturing conditions using anti-p300 antibodies, which also recognize CBP (NM11 from PharMingen). We determined that all of the p300 was removed from cell extracts under the experimental conditions employed, as post-immunoprecipitation supernatants did not contain detectable p300 by Western blot (data not shown), p300/CBP complexes were denatured and used as the source of protein for sequential immunoprecipitation of Ets1 and Smad3 to determine the amounts of Ets1 and Smad3 as well as the acetylation status of Ets1, present in the immunopurified complexes. Ets1 not contained in the p300/CBP fraction was designated as free. All samples were analyzed by Western blotting for the presence of Ets1 using anti-Ets1 antibodies and for the presence of acetylated proteins using anti-acetyl-lysine antibodies.

As presented in Fig. 6D, at the basal or control level (Con) endogenous Ets1 is present in two fractions; one in the p300/CBP fraction and the other, not interacting with p300/CBP, designated as free Ets1 (Fig. 6D). Following TGF-β stimulation, the amount of total Ets1 associated with the p300 complex decreased in a time-dependent manner, whereas the amount of Ets1 in the free fraction increased (Fig. 6D). The acetylated form of Ets1 is preferentially associated with the p300 fraction (Fig. 6E). Following TGF-β stimulation the level of acetylated Ets1 associated with the p300 fraction remains constant (a small decrease at 24 h was observed with the shorter exposures), whereas the level of acetylated Ets1 increases slightly in the free fraction (Fig. 6E). The ratio of acetylated Ets1 to...
The present study establishes for the first time that Ets1 is modified by acetylation and that this modification is rapidly induced by the TGF-β pathway. Furthermore, when overexpressed, Ets1 efficiently antagonizes the profibrotic effects of TGF-β in human fibroblasts. Overexpression of Ets1 did not affect the basal level of collagen type I and other ECM-related genes in unstimulated cells but strongly suppressed the TGF-β-induced COL1A2 promoter activity, type I collagen synthesis and expression of other TGF-β-inducible genes, such as CTGF and TIMP-1. Moreover, Ets1 strongly stimulated MMP-1 and counteracted TGF-β-dependent inhibition of MMP-1. Collectively, our data suggest that elevated expression of Ets1 in fibroblasts fundamentally alters their responses to TGF-β treatment. At present, we cannot distinguish between these two possibilities. In contrast to Ets1, association of endogenous Smad3 with p300 is increased, reaching the highest level 24 h post TGF-β induction (Fig. 6B). Note that endogenous Smad3 preferentially binds to p300, whereas Smad2 remains in the free fraction.

**DISCUSSION**

The present study establishes for the first time that Ets1 is modified by acetylation and that this modification is rapidly induced by the TGF-β pathway. Furthermore, when overexpressed, Ets1 efficiently antagonizes the profibrotic effects of TGF-β in human fibroblasts. Overexpression of Ets1 did not affect the basal level of collagen type I and other ECM-related genes in unstimulated cells but strongly suppressed the TGF-β-induced COL1A2 promoter activity, type I collagen synthesis and expression of other TGF-β-inducible genes, such as CTGF and TIMP-1. Moreover, Ets1 strongly stimulated MMP-1 and counteracted TGF-β-dependent inhibition of MMP-1. Collectively, our data suggest that elevated expression of Ets1 in fibroblasts fundamentally alters their responses to TGF-β in favor of matrix degradation relative to matrix deposition. This novel function of Ets1 is likely to contribute to its pathological role in tumor progression and arthritis.

We further investigated the role of Ets1 in TGF-β signaling using a COL1A2 promoter/reporter construct, which constitutes a relatively well understood molecular model of profibrotic effects of TGF-β. At the basal level, COL1A2 transcription is regulated by Sp1 in cooperation with Fli1 or, less favorably, in combination with Ets1 (25). Upon TGF-β treatment, COL1A2 is stimulated by a multiprotein complex containing Sp1, Smad3, and p300/CBP (37, 39, 49). Although both Ets1 and Smad3 are able to synergize with Sp1 and CDP/p300, Smad3 synergetic interaction requires overexpression or activation by TGF-β (39, 50, 51). Limiting amounts of CBP/p300 in cells (52) provides a competition between formation of various activating or repressing complexes. Our study suggests that TGF-β treatment leads to dissociation of Ets1 from the CBP/p300 complexes (Fig. 6D). As a result, under normal physiological conditions, endogenous Ets1 is not able to interfere with the TGF-β-Smad-dependent stimulation of the COL1A2 gene. In addition, TGF-β-induced dissociation of Ets1 from the CBP/p300 complexes may contribute to the inhibition of the MMP-1 response to TGF-β. We also observed increased acetylation levels of p300/CBP-associated Ets1 in response to TGF-β (Fig. 6). Whether this reflects quantitative and/or qualitative changes in acetylation status of Ets1 remains to be established. Although increased acetylation of Ets1 may correlate with its release from the p300/CBP complexes, additional studies are needed to determine whether these two processes are linked. Acetylated Ets1 may be rapidly targeted by histone deacetylase(s) as has been shown recently for other transcription factors regulated by acetylation (NF-κB) (53). Such rapid deacetylation may account for the reduced acetylation status of Ets1 not complexed with p300/CBP.

In contrast to the Ets1 role under normal physiological conditions, the results of this study indicate that Ets1 overexpression leads to selective inhibition of TGF-β-stimulated genes. Although the full understanding of this process is still lacking at present, several possible explanations based on the existing data and the results of this study can be envisioned. The following, not mutually exclusive mechanisms, may be involved in Ets1-mediated inhibition: competition for the limited amounts of CBP/p300, sequestration of Smad3 by the Jun-Ets1 complexes, and formation of the Ets1-co-repressor complexes. It is noteworthy that under basal conditions, overexpressed Ets1 is by itself a weak activator of the COL1A2 promoter and does not interfere with the stimulatory effect of p300, suggesting that TGF-β signaling is required for Ets1 to manifest its inhibitory function. Although competition between Ets1 and Smad3 for CBP/p300 binding may be involved, we do not think that this is the only mechanism responsible for Ets1-dependent suppression. In transient transfections assays, when p300 is overexpressed, TGF-β-induced COL1A2 stimulation is still abrogated by Ets1 (Fig. 3A), whereas in other experimental systems, overexpression of p300 relieved competition between the activating and repressing factors (34, 54).
Fig. 6. Acetylated Ets1 is preferentially associated with p300. Fibroblasts were treated with TGF-β1 for the indicated periods of time and Co-IP/recapture-IP was performed as depicted on the diagram (A). Equal amounts of protein from each condition (see WB for β-actin in B) were used for IP with p300 antibody. The amount of p300 in each condition was determined by IP/WB (C, left side). Free Ets1 and Smad3 were sequentially immunoprecipitated from the supernatants. The amount of protein in supernatants was quantified by β-actin WB (C, right side). After denaturation, Ets1 and Smad3 were sequentially immunoprecipitated from the p300 complexes (p300/com.). IP from each condition, run on the same gel with p300 complex on the left and free fraction on the right, were analyzed sequentially for Ets1 level (D) and Ets1 acetylation status (E) and in parallel for Smad3 level (F). Normal mouse IgG and rabbit IgG were used for nonspecific controls (NS) for p300 complex and the free fraction, respectively (see right side of panels D-F). Human Ets1 TNT product was used as a positive control. This figure is representative of four independent experiments using two different fibroblast cell lines.
Erks in dermal fibroblasts (47). Consistent with these data, TGF-β does not alter Ets1 phosphorylation either on threonine (Ras-MAPK-dependent) or on serine (mediated by calcium signaling pathways) (43, 44). These findings underscore the importance of the cellular context in regulating functional outcomes of the interactions between transcription factors.

In conclusion, the findings of this study are relevant to rheumatoid arthritis and cancer invasion. Recent studies showed that Ets1 is overexpressed in a subset of synovial fibroblasts in rheumatoid arthritis lesions in vivo (19). Furthermore, there are numerous studies documenting elevated Ets1 expression in tumor and stromal cells in various invasive tumors (1, 64). As our understanding of the role of TGF-β signaling in tumor progression increases, it becomes apparent that the perturbations of the TGF-β signaling pathway play a role in tumor progression without requiring genetic loss of the signaling components of this pathway (65). High levels of TGF-β observed in tumor stroma, together with elevated expression of Ets1 in a subset of stromal cells may result in a microenvironment characterized by both enhanced synthesis and enhanced breakdown of fibrillar collagens. Such a microenvironment has been linked, for example, to malignant ovarian neoplasms (66). The mechanisms of Ets1 transcriptional activation/repression of the TGF-β pathway merits further investigation. These findings will help us to better understand the aggressive behavior of Ets1 overexpressing fibroblasts in arthritis and tumor stroma and may aid in developing novel and specific therapies for these pathological conditions.

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